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The long pentraxin PTX3 as a functional ancestor of antibodies in the immune response to *Aspergillus fumigatus*

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For the Degree of
Doctor of Philosophy

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ABSTRACT

Invasive aspergillosis (IA) is a life-threatening infection amongst immunocompromised individuals primarily caused by the opportunistic fungus *Aspergillus fumigatus* (AF). A key role in the early immune response to AF is played by the complement system, an essential component of the humoral arm of innate immunity that includes three distinct activation pathways: classical, lectin and alternative (CP, LP and AP, respectively). Other soluble pattern recognition molecules (PRMs) are known to cooperate with complement in the handling of AF, including collectins, ficolins and pentraxins. In particular, the long pentraxin PTX3 exerts non-redundant protective roles against AF. PTX3 is a functional ancestor of antibodies: it has opsonic activity towards AF, and enhances recognition, phagocytosis and killing of fungal conidia by immune cells, mainly polymorphonuclear neutrophils (PMNs), via complement and Fc receptors (FcRs) pathways. *Ptx3*-null mice are highly susceptible to IA and treatment with the exogenous protein has therapeutic efficacy in several models of IA in immunocompromised hosts. Furthermore, epidemiologic evidence indicates that variation in the *ptx3* gene is associated with the risk of IA in human stem cell transplant recipients and invasive mold infections in acute leukemia patients.

Here, I characterized the molecular mechanisms underlying the role of PTX3 in the opsono-phagocytosis and killing of AF conidia by human and murine PMNs, both *in vitro* and *in vivo*. I found that PTX3 promotes the selective recruitment of C3b on the wall of AF conidia, by exclusively targeting the AP of complement. Factor H (major inhibitor of the AP) is required for this process, thus pointing to a novel function (activating rather inhibitory) of this complement regulator when combined with PTX3. Also, complement receptor 1 (CR1, primary receptor of C3b), recognizes the PTX3-bound C3b on AF conidia and facilitates their uptake and killing by PMNs. Therefore, I identified a novel functional axis involving factor H, C3b and CR1 that supports the pro-phagocytic and pro-killing activities of PTX3 in antifungal immunity, and opens new vistas on the crosstalk between fundamental humoral components of the innate immune system.

ABBREVIATION LIST

IA: Invasive aspergillosis
AF: Aspergillus fumigatus
PTX3: Pentraxin 3
CRP: C-reactive protein
SAP: serum amyloid P component
CP: Classical pathway
LP: Lectin pathway
AP: Alternative pathway
CCP: complement control protein
MAC: membrane attack complex
MBL: mannan-binding lectin
MASPs: MBL-associated serine proteases
PRM: Pattern recognition molecule
PRR: Pattern recognition receptor
PMN: polymorphonuclear neutrophil
DC: dendritic cell
AM: alveolar macrophage
FcRs: Fc receptors
PAMP: Pathogen associated molecular pattern
ECM: extracellular matrix
TLR: Toll-like receptor
NLR: nucleotide-binding oligomerization domain receptor
RLR: retinoic acid-inducible gene-I (RIG-I)-like receptor
NETs: neutrophil extracellular traps
NHS: normal human serum
DHS: depleted human serum

1. INTRODUCTION

1.1 ASPERGILLOSIS

The incidence of fungal infections has steadily increased over recent years, and invasive forms of these pathogens pose a severe threat to a growing number of individuals, mostly those experiencing conditions of immune suppression and deficiency [9,10]. Among the wide spectrum of fungal pathogens that are able to infect a susceptible host, the saprobic environmental ascomycetes of the *Aspergillus* genus stand out as a paradigm of environmental microbes that turn into opportunistic pathogens under “permissive” host conditions [11]. *Aspergillus* species are ubiquitous soil molds, perhaps the most common amongst those encountered by humans. More than 650 species of *Aspergillus* have been documented, and around 40 have been reported to be human pathogens [12]. The most prevalent is *Aspergillus fumigatus*, which accounts for approximatively 90% of invasive aspergillosis (IA) cases [13-15], followed by fungi of the so called non-*fumigatus* species, such as *A. flavus*, *A. niger*, *A. terreus*, *A. ustus*, and *A. lentulus* [16,17]. These species are also responsible for a number of livestock infections (e.g., in cattle and horses [18]) and crop infestations [19]. In humans, *Aspergillus* gives rise to a broad spectrum of clinical manifestations, ranging from non-invasive infections, such as allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) and aspergilloma, to invasive forms of the disease [20,21]. In a recent meta-analysis the global burden of non-invasive aspergillosis (including ABPA in asthma and cystic fibrosis patients) has been estimated as ~5 million cases per year, and as many as 10 million individuals have been figured to be at risk of IA worldwide, with a great impact on healthcare systems [22]. This picture, however, is likely incomplete and the numbers reported above might well be underestimated, due to limitations in the currently available diagnostic tools [23]. IA is the most severe form of aspergillosis, with a global incidence of more than 300,000 cases per year and a mortality rate as high as 95% in the worst clinical settings [16,24]. In the course of a typical IA infection, following invasion of the host at the initial sites of insult (i.e., the lung alveoli), the pathogenic fungus undergoes haematogenous dissemination to distant organs, including the central nervous system, liver, spleen and skin, whose function is often irreversibly compromised. As prototypical opportunistic pathogens, *Aspergillus* species almost exclusively hit immunocompromised individuals, such as patients with

hematological malignancies, recipients of bone marrow stem cell and solid organ transplants, patients administered with corticosteroids for treatment of autoimmune diseases, and individuals with genetic immunodeficiencies (e.g., chronic granulomatous disease, CGD) or infected with human immunodeficiency virus [25,26]. It is therefore apparent that the host immune landscape plays a fundamental role both in the onset and outcome of the disease. The currently available therapeutic options for the treatment of IA are largely based on either individual or combined administration of three classes of antifungal drugs, namely triazoles, amphotericin B, and echinocandins [27]. All of them, however, have been reported to have significant drug-drug interactions, and both acute and chronic side effects. Furthermore, no new classes of antifungals have been commercialized since the launch of the echinocandins in 2001, with the pharmaceutical pipeline of developing novel antifungal drugs being about to run dry [28]. Therefore, new therapies are needed that are based on a better understanding of the host/pathogen interplay in IA pathogenesis to be used in conjunction with or replace existing treatments.

1.2 *ASPERGILLUS FUMIGATUS*

Aspergillus fumigatus is a saprophytic fungus belonging to the *fumigati* section of the *Aspergillus* subgenus. This section includes 9 anamorphic and 24 sexually reproducing species [29,30]. *Aspergillus fumigatus* is a ubiquitous mold that undergoes several morphogenetic transitions in response to a number of triggering events, including virulence factors, biofilm formation and, in the case of an infection, host environment (see Figure 1). This mold spreads in the environment in the form of metabolically inactive airborne spores (conidia), which are made and released by conidiophores in a process known as conidiation or sporulation. Under permissive conditions (e.g., suitable temperatures, availability of nutrients), conidia first undergo symmetric growth into larger morphotypes (swollen conidia) that is marked by substantial changes in size, structure and composition of both intracellular compartments and cell wall, then generate asymmetric, tube-like structures (germlings) that progress into polarized filamentous hyphae (mycelium) [31]. Columnar, smooth-walled, 300 μm long conidiophores are eventually formed on the fungal mycelium that are responsible for generation and release of new conidia [31]. *Aspergillus fumigatus* has a remarkable sporulating capacity, which results in the ubiquitous occurrence of conidia in the air at relatively high concentrations (1 to 100 spores per m^3) [32,33]. Conidia are green, rough-surfaced spheres with a small diameter (2-3 μm) and a complex cell wall

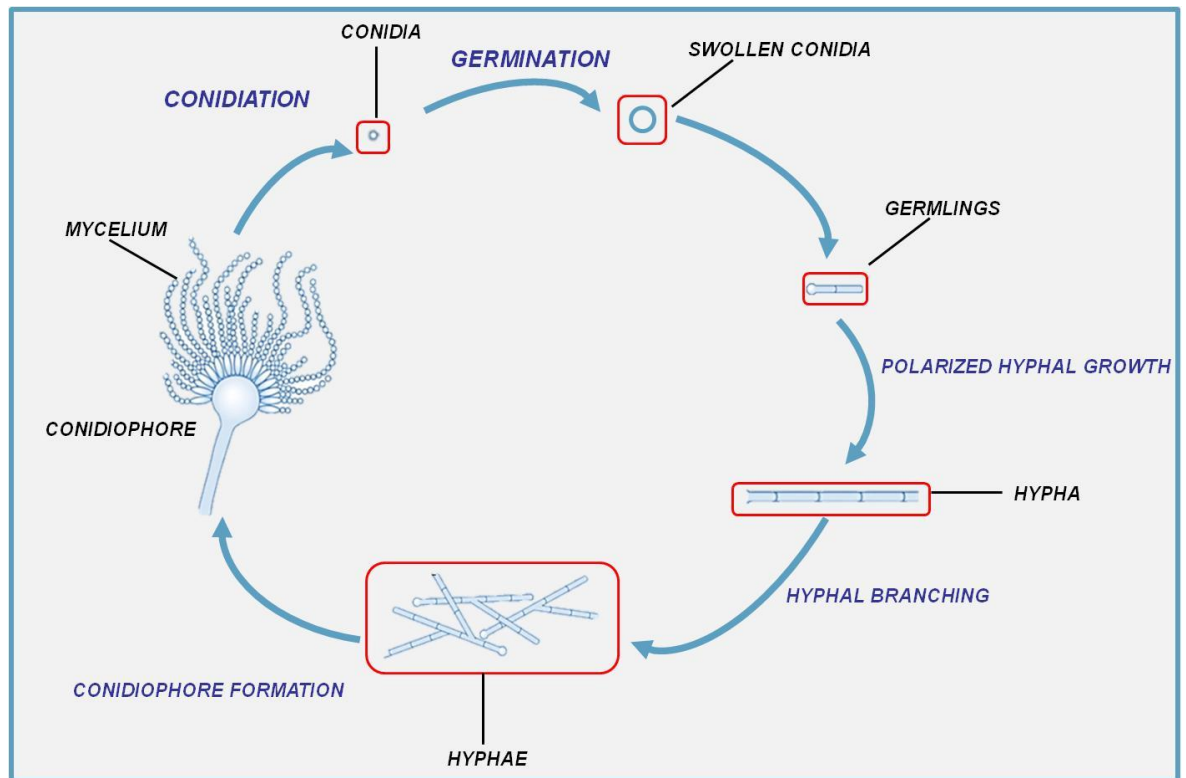


FIGURE 1. The life cycle of *A. fumigatus*. *A. fumigatus* spreads in the environment in the form of airborne spores (conidia). Under permissive conditions, conidia (~3 μm across) can initiate a germination program, first evolving into swollen conidia (~6 μm across), then generating germinals, and filamentous hyphae (mycelium). These eventually make conidiophores, which can produce and disseminate new conidia (adapted from Shapiro *et al.* [23]).

made up of several components, including polysaccharides, proteins, lipids, melanins and other pigments. Polysaccharides are the most abundant components of the cell wall, and mainly comprise β - and α -1,3-glucans, chitins and galactomannans [34]. These associate with proteins through both covalent and non-covalent interactions, forming an intricate 3D network with regions of rigid structure neighboring others resembling amorphous matrices [35]. In this regard, electron microscopy investigations have shown the occurrence of distinct layers of polysaccharides in the conidial wall. The innermost layer (i.e., close to the plasma membrane) mostly comprises β -1,3-glucans, linked to chitin via β -1,4-linkages, whereas the outermost (i.e., that underlines the melanin coat; see section 1.2.2.) is rich in α -1,3-glucans. In between the two, a fibrillar region occurs that displays a mixed polysaccharide composition with interspersed proteins and galactomannan chains [36]. The cell wall undergoes profound modifications in structure and composition as *Aspergillus fumigatus* conidia germinate and become hyphae. Dynamics and plasticity of the cell wall are necessary for fungal growth, adaptation to changing environmental conditions, and

protection from external stressors. Furthermore, the cell wall provides mechanical support and shape to the fungal cell, mediates adherence to surfaces, and contains most of the enzymes involved in the generation of biofilms [37-39].

1.2.1 INFECTION AND INVASION

A. fumigatus conidia are abundantly generated on and released by conidiophores within a few hours from exposure of the filamentous mycelium to air/water interfaces [40]. Up to hundreds of newly made conidia are inhaled daily by humans, and due to their small size, they can pass through the upper airways and reach the lower respiratory tracts (i.e., lung alveoli) [5]. The first line of defense against the entering spores is mechanical in nature, and provided by the rhythmic beating of ciliated cells in the lung epithelium (mucociliary movements), mostly active in the upper respiratory tracts [41]. However, in cases of impaired mucociliary clearance (e.g., in cystic fibrosis, asthma, bronchiectasis), conidia are spared, at least partly, from mechanical disposal and able to penetrate into the alveoli, where they are faced by both resident and recruited innate immune cells, including alveolar macrophages, dendritic cells and polymorphonuclear neutrophils (Figure 2; see section 1.3). It is the immunological status of the host that then dictates the final outcome of the infection, whether conidial propagules can colonize and spread in a susceptible host or are recognized and eliminated by the effector cells of an immunocompetent individual [42]. Indeed, in the absence of a proper immune response, *A. fumigatus* conidia can find a favourable ground to swell, germinate, and polarize into hyphae. Germination begins in the alveolar space, mostly in pulmonary epithelial cells, and subsequently germlings and hyphae invade the surrounding tissues, including the endothelium. In this regard, it is increasingly apparent that the lung epithelium (traditionally regarded as a mere physical barrier) is equipped with molecules and mechanisms common to those of the innate immune system, and participate in the early recognition and, possibly, disposal of *A. fumigatus* [43,44]. Alveolar epithelial cells (mostly type II pneumocytes) are the first to encounter invading conidia. Similar to macrophages and neutrophils, these cells have phagocytic and killing activities, although these are less effective compared to those of the innate immune cells [45]. Most importantly, conidia that have been internalized by epithelial cells can survive and even germinate in the intracellular phagolysosome [46]. In this respect, the melanin layer of the fungal wall is believed to inhibit cell apoptosis [47] and phagolysosome acidification [48], thus contributing to a favourable microenvironment

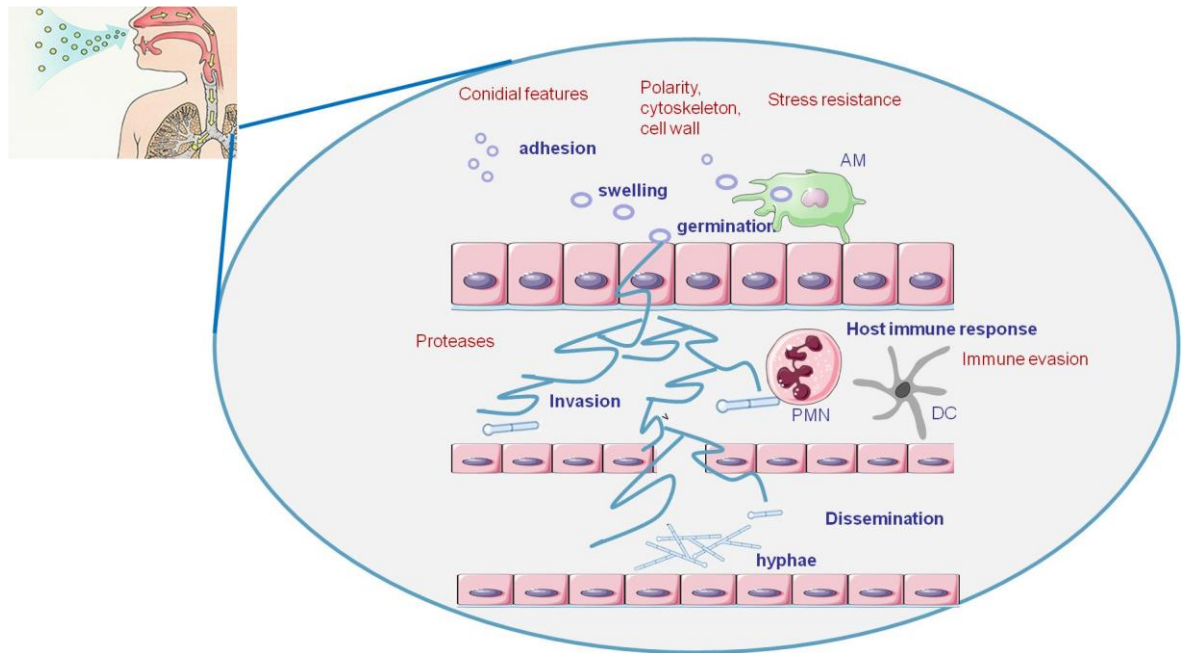


FIGURE 2. *A. fumigatus* infection and dissemination. *A. fumigatus* conidia that have escaped mucociliary disposal land in the alveolar cavities, where they can adhere to the lung epithelium, and start the germination program with colonization of the surrounding tissue. Early defense against the invading mold is provided both by humoral (not displayed in the figure) and cellular components of the innate immune system. A major role in this regard is played by tissue resident alveolar macrophages (AM) and dendritic cells (DC), and tissue-recruited polymorphonuclear neutrophils (PMN). If not eliminated, germinating conidia can develop to hyphae, invade the lung parenchyma, breach the endothelial wall and eventually disseminate to distant organs. Some of the factors contributing to pathogenicity of *A. fumigatus* are indicated in red (see main text for more details). Adapted from Krappmann [5] .

to survival and growth of the pathogen (see section 1.2.2). Upon breaching into the vessel wall, *A. fumigatus* hyphae become angioinvasive, and disseminate to distant organs and tissues via the blood stream [49]. Yet, the actual mechanisms of angioinvasion and invasion of distant tissues, most notably the brain, are still unclear. Fungal infections of the central nervous system (FIs-CNS) have become significantly more common over the past 2 decades. In states of blood-brain-barrier (BBB) perturbation, such as trauma, surgery, or inflammation (e.g., activation of microglia, endothelium, with cytokine/chemokine release), the BBB permeability increases, which facilitates penetration of the fungus into the brain. The pathogens therefore gain access to the cerebral parenchyma and locally proliferate causing brain inflammation [50]. In this regard, *A. fumigatus* is responsible for the majority of brain abscesses in immunocompromised patients [51,52], where it reaches the CNS via haematogenous or direct spread (i.e., from the paranasal sinuses, mostly in the course of sinusitis and mastoiditis) [53,54]. Haematogenous spread is made possible by the

interaction of *A. fumigatus* with components of the vessel endothelium (see section 1.2.2 and [55]). In this regard, the circulating pathogens in the blood must first be arrested in the brain microvasculature, then transmigrate into the brain parenchyma across the BBB. Two mechanisms have been described for *A. fumigatus* to cross the BBB: transcellular and paracellular migration [56,57], where both involve initial infection of phagocytes in the blood (mostly macrophages and neutrophils), and hijacking the infected leukocytes for BBB permeation [50]. Indeed, once internalized, the pathogen may actively manipulate the phagocyte to promote migration to the brain, where it adheres to the luminal side of brain capillaries and crosses the BBB, either paracellularly or transcellularly [58]. In this regard, *Aspergillus* species can make and release mycotoxins, which inhibit phagocytosis and increase conidial resistance to opsonisation (see section 1.2.2 and [50], besides being able to damage and kill microglia, astrocytes, and neurons [50]).

Humans have evolved immune effector mechanisms to rapidly and effectively recognize and kill invading fungal pathogens. These mostly comprise both the humoral and cellular arms of the innate immune system, with additional and complementary roles played by adaptive immunity. Initial sensing of *A. fumigatus*, and in general of any invading microbe, by a mammalian host involves the interaction of evolutionary conserved molecular signatures, named pathogen associated molecular patterns (PAMPs), with germline-encoded recognition molecules, collectively defined as pattern recognition receptors (PRRs). Given that any microbe is endowed with a number of PAMPs that are recognized by a variety of PRRs, multiple PAMP/PRR interactions normally occur at the host/pathogen interface. These events are deciphered by the mammalian cell and translated into specific signaling programs that ultimately lead to “tailored” immune responses both at the cellular and molecular level (e.g., activation/repression of distinct cell subsets, induction/inhibition of selected soluble molecules, including cytokines and chemokines) [5]. Major cellular and molecular players in the orchestration of the immune reaction to *Aspergillus fumigatus* are described in more details in section 1.3.

1.2.2 VIRULENCE FACTORS AND IMMUNE EVASION STRATEGIES

In spite of the increasing incidence of invasive aspergillosis, pathogenesis of *A. fumigatus* infections is still poorly understood. In this regard, a number of properties have been described that support and facilitate environmental adaptation of this mold, and virulence

factors have been identified that allow persistence and spreading in the human host, including mechanisms of evasion of the immune response (Figure 3). *A. fumigatus* is a thermophilic fungus, which can grow at temperatures as high as 55 °C and survive at even higher temperatures, up to 75 °C [59,60]. This thermal adaptation is mediated by heat shock proteins (Hsp70 and 40), and is crucial for fungal growth and metabolic activity in as diverse environments as soil (to support decay of the organic matter) and mammalian hosts (to allow germination and invasion) [59]. Additional mechanisms have been described that support resistance of *A. fumigatus* to external stressors, including osmotolerance [61], unfolded protein response [62], and adaptation to varying external pH [63] as well as hypoxic conditions [64]. Furthermore, this mold can produce several enzymes (including the flavohemoglobins FhpA and B, and the S-nitrosogluthathione reductase GnoA) with detoxification activity towards the reactive nitrogen intermediates (RNI) generated by macrophages and neutrophils [37]. However, the actual role of these enzymes in fungal virulence has been recently questioned by the observation that *fhpA*-, *fhpB*-, and *gnoA*-deficient strains of *A. fumigatus* display wild-type pathogenicity in a murine model of IA [38]. The small size of *A. fumigatus* conidia (2-3 µm across) is *per se* strategic to the pathogenicity of the fungus in that it allows deep penetration into the lower airways, where mucociliary clearance is less efficient [65,12]. In addition, the conidial wall is equipped with molecules that contribute to protecting the microbe from hostile environments and facilitating its adhesion to host cells and tissues. In this regard, wall-associated sialic acid, either harvested from the host or *de novo* synthesized by the fungus, has been shown to mediate adhesion of conidia to the extracellular matrix (ECM) of host tissues, through its interaction with selected ECM components, most notably fibronectin [66]. This mechanism is believed to promote fungal infection and invasion, since the extent of conidial wall sialylation has been observed to correlate with pathogenicity of the mold [67,68]. Also, *A. fumigatus* has been shown to recruit plasminogen onto its surface, where this mechanism is believed to promote disruption of and penetration into the host tissue via the fibrinolytic pathway [34]. Moreover, a layer of dihydroxynaphthalene (DHN)-melanin has been described in the conidial wall that protects the fungus not only from ultraviolet light (in the external environment) but also from enzymatic lysis and oxidative damage, where both processes take place in the phagolysosome of host cells (primarily neutrophils and macrophages) and are mediated by hydrolytic enzymes and reactive oxygen species (ROS) that are abundant therein [68]. This paradigm, however, has been recently revisited in a report on the identification of a C-type lectin receptor (MelLec or CLEC1A) that

recognizes the naphthalene-diol unit of DHN-melanin in fungal conidia, and provides host protection (rather than infection, as one would argue based on the proposed ROS scavenging activity of DHN-melanin in the phagolysosome) in a mouse model of disseminated *A. fumigatus* infection [69]. MelLec was found on endothelial cells and an epithelial-like subset of these cells in mice, and is expressed by human myeloid cells. Interestingly, in the same study an association has been described between a single nucleotide polymorphism of this receptor and the susceptibility of stem-cell transplant recipients to disseminated *Aspergillus* infections, a finding that points to melanin as an immunologically active component of the fungal cell wall (see also section 1.4.6).

A. fumigatus can synthesize and release mycotoxins, metabolites with cytotoxic activities that impart the fungus with high virulence in the infected host [70]. The best known of them is perhaps gliotoxin, a metabolite of the epipolythiodioxopiperazine family [71] that inhibits phagocytosis and induces apoptosis in macrophages, and impairs activation of both T and B lymphocytes [72,73]. Gliotoxin can also inhibit the mucociliary movements, which results in prolonged residence of the fungus in the lung epithelium, a condition that favours adhesion and invasion [71].

The outermost layer of *A. fumigatus* conidia is mostly comprised of hydrophobic proteins (i.e., hydrophobins), which self-assemble on the conidial surface into regularly arranged rodlets, a structure that favours air buoyancy and dispersion of the conidia by air streams [74]. RodA is the most important and best characterized *A. fumigatus* hydrophobin and shown to play important roles at the conidia/host interface [75]. Indeed, RodA rodlets enhance adhesion of conidia to selected host proteins (i.e., albumin and collagen) [76], and inhibit the innate immune response to these fungal morphotypes [65,12]. RodA is poorly recognized by the innate immune system, and masks PAMPs, (most notably β -1,3-glucans and the α -mannose residues in the galactomannans) to recognition by the PRRs dectin-1 and dectin-2, respectively [77,78] (see section 1.3). This evasion mechanism prevents both mounting and development of inflammatory responses within the infected lung in response to daily inhalation of conidia [79]. In line with this, conidia derived from RodA-deficient strains of *A. fumigatus* are more readily phagocytosed than their wild type counterparts by human macrophages *in vitro* [75]. The RodA rodlets rapidly disassemble as conidia swell and germinate, therefore the polysaccharide-containing inner layers of the cell wall become accessible to immune recognition at late stages of the infection only, when the fungus has already colonized the lung alveoli [75,80].

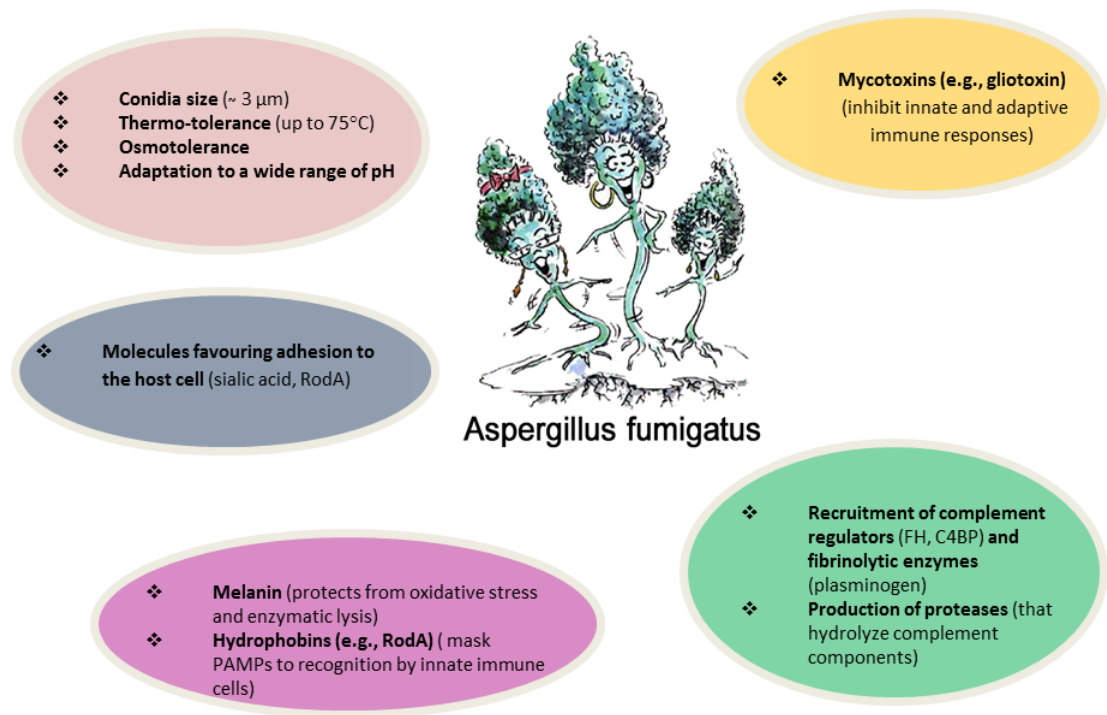


FIGURE 3. Major virulence factors of *A. fumigatus* and their proposed mechanisms of action.

Recently, Asp2, a protein allergen expressed by *A. fumigatus*, has been shown to recruit human plasminogen and make use of the newly generated plasmin (i.e., from proteolytic activation of plasminogen by tissue-type plasminogen activator) to degrade fibrin and fibrinogen. As a consequence, conidia-bound plasmin have been reported to damage human lung epithelial cells, induced cell retraction, and cause matrix exposure. Therefore, by contributing to disruption of the human lung epithelium, Asp2 plays a role in the early steps of fungal infection, likely promoting penetration of the pathogen in the lung tissue [81].

As an additional virulence strategy, *A. fumigatus* is endowed with the capability to recruit on its surface negative regulators of the alternative and classical/lectin complement pathways, such as factor H (FH) and factor H-like protein 1 (FHL-1), both **via Asp2 [81]**, and C4b-binding protein (C4BP), respectively, which provides a way to escape the complement system mediated immune response (see section 1.4.6) [82]. Furthermore, *A. fumigatus* can synthesize and secrete proteases (e.g., Alp1) that target for proteolytic degradation complement components, like C3, which are essential for opsonisation and phagocytosis of the mold by innate immune cells [83]. This highlights the structural

complexity of the fungal wall, which is not only permeable to small metabolites (e.g., gliotoxins), nutrients and waste products, but also allows transit of proteases for extracellular inactivation of immune effector mechanisms (see Figure 3 for a graphical summary).

1.3 INNATE IMMUNITY AND *A. FUMIGATUS*

The innate immune system is an evolutionary conserved system acting as a first line of defense against exogenous and endogenous threats, including pathogenic infections, tissue damage and cancer [84]. Destruction and clearance of invading pathogens, including *A. fumigatus*, requires the complex coordination of multiple pathways of both innate and adaptive immunity. The innate immune response precedes and orientates the highly specialized adaptive reaction, and supports the long-lasting immunological memory of adaptive immunity. Major cellular components of the innate immune system comprise macrophages (MØ), dendritic cells (DCs), polymorphonuclear neutrophils (PMNs), natural killer (NK) and innate lymphoid cells [85,84]. These are all equipped with receptors (cell-associated PRRs) that enable recognition of exogenous entities (non self) and damaged host cells and tissues (modified self) via their PAMPs and danger associated molecular patterns (DAMPs), which are not present, or are poorly expressed, on healthy host cells and tissues (self). Recognition of microbes and microbial moieties is also mediated by soluble pattern recognition molecules (PRMs) that cooperate with cell-associated PRRs to mount and orchestrate an appropriate immune response [86]. The soluble components of the innate immune system include complement, ficolins, collectins and pentraxins, and will be the subject of other sections. The inflammatory reaction that follows an immune recognition event is fundamental for effective and long-lasting immunity, and is finely controlled in time and space upon resolution of the infection or elimination of the damaged cells/tissues, a mechanism that spares proximal healthy tissues from unwanted damage, like that occurring in autoimmune or autoinflammatory diseases [87].

1.3.1 THE CELLULAR ARM

The cellular arm of the innate immune system is composed by different cell types, as described above, which express a number of PRRs strategically located on the cell

membrane, in endosomal compartment and the cytoplasm. These includes Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD) and retinoic acid-inducible gene-I (RIG-I)-like receptors (NLRs and RLRs, respectively), scavenger receptors, dectins and components of the inflammasome [88,89]. The inflammatory response that follows an infectious insult actively recruits immune cells to the infection site via several mechanisms, including generation of a gradient of chemoattractants, increased permeability of the vessel endothelium, and local augmentation of the blood flow [90]. When activated in the course of an inflammatory response, these cellular effectors coordinately work to phagocytose and kill microbes, clear cellular debris and control the initial wave of inflammation, thus contributing to the maintenance of tissue homeostasis. These cells provide a first line of defense against many microorganisms and are essential for the control of common bacterial and fungal infections, including those mediated by *Aspergillus fumigatus*. They also play a crucial role in the initiation and subsequent control of adaptive immune responses, and participate in the clearance of pathogens that have been targeted by an adaptive immune response (e.g., in antibody-dependent phagocytosis and cytotoxicity). However, in certain cases, the innate immune system is unable to deal with the infection, and so activation of an adaptive immune response becomes necessary [91]. In these cases, the innate immune cells can instruct the adaptive immune system about the nature of the pathogenic challenge. They do so through diverse mechanisms, including the expression of costimulatory molecules on the surface of specialized antigen-presenting cells and production of distinct chemokines and cytokine profiles [92].

1.3.1.1 *POLYMORPHONUCLEAR NEUTROPHILS*

PMNs are the most abundant granulocytes and the most numerous white blood cell population in the vast majority of mammals [93]. Together with basophils and eosinophils, PMNs form the polymorphonuclear family of cells and are normally found in the bloodstream. Derived from bone marrow stem cells, they are short-lived, highly motile, and phagocytic cells that can enter regions of tissue which are not or poorly accessible to other cell types [93]. Indeed, during the acute phase of inflammation, particularly as a result of bacterial and fungal infections, but also in the context of cancerogenesis, neutrophils are one of the first-responder, inflammatory cells to migrate towards the site of inflammation [93]. They extravasate from blood vessels and migrate to the infected/inflamed tissue, following a hierarchical pathway of intermediate endogenous

chemokines (e.g., Interleukin-8 (IL-8), also now known as CXCL8), and end-target chemoattractants, including bacterial or fungal peptides (e.g., N-formyl-methionyl-leucyl-phenylalanine, fMLP) and complement activation products (e.g., C5a, see section 1.4) [94-96]. The migration process is normally accomplished within minutes, and tissue-recruited PMNs are extremely efficient in recognizing and phagocytosing invading microorganisms. Both surface-associated and intracellular PRRs are involved in the recognition of microbial PAMPs by PMNs, and coordinately induce intracellular signals that lead to full pathogen-killing capacity. This is mostly due to an extraordinary armamentarium of antimicrobial proteins, peptides, proteases, and components of the respiratory burst (e.g., ROS). This collection of “weapons” can be primed and “fired” by the engagement of a wide range of membrane-bound receptors for microbial products, soluble mediators of inflammation, endothelial adhesion molecules and extracellular matrix proteins [97,98]. Responsiveness of PMNs to microbial PAMPs is amplified by intracellular vesicle trafficking circuits [93]. In this regard, the secretory vesicles of PMNs act as intracellular reservoirs of membrane-associated receptors, which are promptly mobilized in response to microbes/microbial moieties and a wide variety of inflammatory stimuli [99,93]. For example, the membrane of these vesicles is rich in membrane-bound PRRs, adhesion molecules and innate effector molecules, including the β 2-integrin CD11b/CD18 (also known as macrophage antigen-1, Mac-1, or complement receptor 3, CR3) [100], complement receptor 1 (CR1, or CD35) [101], the lipopolysaccharide (LPS)/lipoteichoic acid (LTA) receptor CD14 [102], Fc γ receptors (Fc γ R), such as Fc γ IIIR (CD16) and Fc γ IIR (CD32) [102], and the metalloprotease leukolysin [103], all of which translocate to the plasma membrane upon vesicle exocytosis. In this regard, complement receptors and Fc γ R are known to co-localize in phagocytic cups (cup-shaped invaginations of the cell membrane that close at their distal margins to form phagosomes during phagocytosis), and functionally cooperate to promote engulfment and phagocytosis of microbes and microbial particles [104]. As an additional example of cell activation via redistribution of molecules between plasma and vesicle membranes, mobilization of secretory vesicles is accompanied by the shedding of L-selectin from the PMN cell surface, and the combined changes in plasma membrane allow neutrophils to establish firm contacts with the activated vascular endothelium *in vivo* [105]. The controlled trafficking of intracellular components is therefore largely responsible for the functional plasticity of PMNs, and their transformation from “passive” circulating cells to potent effectors of the innate immunity. In addition to secretory vesicles, which are the first intracellular compartments to undergo mobilization, gelatinase,

specific and azurophil granules are also exocytosed in the course of PMN activation, with a specific temporal and spatial hierarchy, as indicated by reports on the *in vivo* migration of neutrophils into the skin during acute aseptic (or sterile) inflammation [106]. Regulated exocytosis of granules enables PMNs to deliver their arsenal of cytotoxic granule proteins in a targeted manner, thus preventing or limiting widespread damage to host tissues.

Besides secretory and phagocytic effector functions, PMNs can also extrude neutrophil extracellular traps (NETs), a web of fibers composed of chromatin and serine proteases that trap and kill microbes extracellularly [107]. It has been suggested that NETs provide high local concentrations of antimicrobial components and their contribution to the elimination of microbes is independent of phagocytic uptake. Neutrophils are unique in their capacity to adapt to the microenvironmental conditions of the tissue, and contribute both to initiation and resolution of inflammation [108]. In this respect, they can establish close molecular crosstalks with other cell types, and are believed to coach DCs, monocytes and lymphocytes and help them decide whether to initiate and maintain an immune response [93,109].

The classical paradigm of host defense against *A. fumigatus* implies initial phagocytosis of conidia by resident macrophages and neutrophils. These latter cells are recruited to the site of infection within a few hours from entry of the fungal propagules, and play essential roles in recognition and killing of different *A. fumigatus* morphotypes, including resting and germinating conidia, and filamentous hyphae [110]. Killing of *A. fumigatus* involves a number of mechanisms, including production of ROS, formation of NETs, release of the microbicidal components from intracellular granules (degranulation) and phagocytosis of the fungal particles (via generation of phagocytic cups) [111]. In this regard, lactoferrin, a secondary granule protein in neutrophils, has been shown to inhibit *A. fumigatus* growth via chelation of iron ions, which are essential for fungal metabolism [112]. Furthermore, neutrophil elastase is an important lytic enzyme in the degradation of NET-entrapped *A. fumigatus*. [113]. Of major relevance to this study, PMNs store in their specific granules packages of pre-made long pentraxin PTX3 [98], a soluble PRM with non-redundant protective roles in IA that is immediately released at sites of infection upon PMN degranulation (see section 1.5).

Defects either in number, recruitment or function of PMNs have been associated to increased susceptibility to IA, which highlights the importance of these cells in the containment of *A. fumigatus* infections. For example, congenital (e.g., mutations in the

neutrophil elastase 2 gene, ELA2) and acquired neutropenia (i.e., abnormally low concentration of neutrophils in the blood, e.g. due to anti-neutrophil antibodies) is long known to be a major risk factor for IA [114,115]. Inherited defects in leukocyte adhesion molecules, including the β chain (CD18) shared by Mac-1 (CD11b/CD18) and lymphocyte function-associated antigen 1 (LFA-1, or CD11a/CD18) integrins, are associated with reduced endothelial adhesion and extravasation, ultimately leading to decreased recruitment of PMNs to the infection site [116]. Chronic granulomatous disease (CGD, also known as Bridges–Good syndrome) is a paradigmatic example of PMN functional defects that predispose to *A. fumigatus* infections, and comprises a diverse group of hereditary diseases in which production of reactive oxygen compounds (with major regard to the superoxide radicals generated by NADPH oxidase) is impaired or compromised [117]. Another clinical condition that results in impaired *A. fumigatus* killing is the Chediak-Higashi syndrome, an autosomal recessive disease characterized by defective fusion of phagosomes and lysosomes [118]. Most importantly, in an era of extensive iatrogenic immune suppression, chemotherapeutic ablation of PMNs (e.g., by means of cyclophosphamide), and their functional impairment (e.g., by cortisone acetate) have become major risk factors for IA in a growing number of individuals [119-122].

1.3.1.2 *ALVEOLAR MACROPHAGES*

Macrophages play a key role in the innate recognition of pathogens. Blood monocytes derive from bone marrow precursors (i.e., monoblasts and promonocytes), and mature into macrophages in the peripheral tissue [123]. Resident populations of macrophages are found at all portals of entry for pathogens such as the gut, lung or skin, as well as in the stroma of organs central to host defense such as the liver, spleen, bone marrow and lymph nodes [124].

Macrophages recognize pathogens either directly through cell-associated PRRs or indirectly by interacting with soluble PRMs and antibodies that bind (i.e., opsonise) the microbial surface. Expression of PRRs varies across macrophages (i.e., depending on their functional state) and is also subject to regulation by cytokines and other stimuli [125]. Macrophage-associated PRRs, in addition to mediating pathogen recognition, can also determine the mechanisms and routes of cellular uptake as well as the subsequent macrophage responses. Multiple PRRs and opsonic receptors (i.e., that sense pathogen-

bound opsonins) can recognize a single pathogen, so internalization and activation of the subsequent inflammatory response are likely to be the integrative outcome of these interactions [126]. Recognition of a microbe by a macrophage generally leads to its internalization and killing. In this regard, macrophages express a multitude of PRRs and have a prodigious capacity to ingest and eliminate microbes [127].

Macrophages need to be activated to optimize their capacity to control an infection, and both microbial products and cytokines contribute to their activation. In response to intact organisms and microbial products changes in production and secretion of pro- and anti-inflammatory molecules occur, with a concomitant increase in the expression of co-stimulatory molecules and PRRs, like, for example, scavenger receptors [128]. Activated macrophages produce a large variety of molecules, including microbicidal peptides, opsonic complement components, cytokines, chemokines and major histocompatibility complex (MHC) proteins involved in antigen presentation. Upon resolution of the infection, activated macrophages need to be “inactivated” to minimize damage to the host tissue, and this is largely orchestrated by “anti-inflammatory” cytokines, such as IL-10 and type I interferons [129].

As described above, following inhalation airborne conidia of *A. fumigatus* can pass the long airway routes with the respiratory air flow, finally landing in the alveoli. In these deep parts of the lung tissue they encounter the alveolar macrophages (AM) [130]. These are placed beneath the alveolar surfactant film, and account, in healthy, untreated mice, for more than 90% of leukocytes in the bronchoalveolar lavage [131]. The initial contact of conidia with the host immune system can lead to direct phagocytosis. Although this process is described to require a profound reorganization of the macrophage microfilamental system that leads to uptake (i.e., in the phagocytic cup) and killing (i.e., in the acidic phagolysosomal compartment) of the fungus [132,133], relevant PRRs remain poorly defined. In this regard, as previously discussed (see section 1.2.2), resting conidia of *A. fumigatus* are fully capped by a surface hydrophobic layer of RodA, which masks immune-triggering structures of the fungal wall to recognition by most PRRs [75,134]. Until the recent discovery of MelLec as a melanin-sensing receptor [69], the only cell-associated molecule proposed to recognize the conidial surface was dendritic cell-specific intercellular adhesion molecule 3-grabbing non integrin (DC-SIGN). This molecule is mainly expressed on dendritic cells, and is also present on macrophage subsets, where it binds *A. fumigatus* conidia in a galactomannan-dependent manner, leading to

internalization of the spores [135]. This notion, however, is counterintuitive, given that galactomannans are not normally exposed on the conidial surface, rather they are shielded by the RodA rodlets [136]. Nonetheless, the hydrophobic outermost layer of *A. fumigatus* conidia is not a “continuum” (see “Virulence factors and immune evasion strategies”), and it is likely that certain inner components of the fungal cell wall, like galactomannans, might be accessible to PRRs, at least in conditions of increased permeability of the RodA layer (i.e., when resting conidia rescue their metabolism and initiate the germination program). This concept likely applies to recognition of melanin by the newly identified MelLec receptor [69].

If not internalized and thereby eliminated by AMs, the *Aspergillus* spores take advantage of the humid and nutrient rich milieu of the lung to start swelling, the initial step of the germination process that is associated with a dramatic reorganization of the conidial wall, which exposes, among other molecules, β -1,3-glucans [137,138]. These polysaccharides are the major targets of Dectin-1, a C-type lectin-containing receptor that is expressed on many cell types, including macrophages, monocytes, neutrophils and a subset of T cells [139]. Recognition of swollen conidia or even germlings by Dectin-1 on AMs leads to phagocytosis and synthesis of different pro-inflammatory cytokines, like TNF- α , IL-6 and IL-18 [140]. The uptake of fungal morphotypes by AMs is enhanced by opsonisation of conidia or hyphae by soluble PRMs, as demonstrated by evidence from opsonin-deficient mice that are susceptible to *A. fumigatus* infections. This has been reported, for example, for the long pentraxin PTX3 [141], the collectin surfactant protein D (SP-D) [142] and the complement factor C3 [143].

The microbicidal activity of AMs is extremely potent, with up to 90% of the spores killed within 30 hours of infection [124]. Although AMs can readily phagocytose resting conidia, intracellular swelling of the spores is a prerequisite for their ultimate killing in the phagolysosome, indicating an important contribution from intracellular PRRs and PRMs [144] (see below). Other than phagocytosing and killing fungal elements, AMs are also a major source of soluble factors that can further enhance the immune reaction to the fungal insult. In this regard, recognition of *A. fumigatus* PAMPs by either TLRs [127] or Dectin-1 [140] leads to uptake of the spores. When in the cell, intracellular receptors, like NOD-2, a member of the NLR family [145], initiate myeloid differentiation primary response 88 (MyD88)-dependent and -independent, and mitogen-activated protein kinase (MAPK) signaling pathways, leading to the synthesis of pro-inflammatory cytokines like TNF- α , IL-

12, IFN- γ , IL-18, IL-6, IL-1 β , GM-CSF (Granulocyte-macrophage colony-stimulating factor), MIP-1 α (Macrophage Inflammatory Protein-1 α), MCP-1 and -2 (Monocyte chemotactic protein 1 and 2) and KC (Keratinocyte chemoattractant) in the mouse [146,119]. Induction of these genes is controlled by a number of transcription factors, including NF- κ B [147], AP-1 [148] and IRF3 [149]. It is worth nothing here that the expression of several cytokines changes significantly under immunosuppressive conditions (e.g., administration of corticosteroids) thus contributing to the immune “milieu” becoming “permissive” [146,119].

1.3.1.3 *DENDRITIC CELLS AND INITIATION OF ADAPTIVE IMMUNITY*

Similar to PMNs and AMs, recognition and phagocytosis of fungal particles by dendritic cells (DCs) is mediated by a number of PRRs. In this respect, Bozza *et al.* [150] have shown that mannose receptor (MR) and C-type lectin receptors (CLRs) with specificity for galactomannans initiate the uptake of *A. fumigatus* conidia by DCs. Also, CR3 together with Fc γ RII and III, mediate the engulfment of unopsonised hyphae [151]. Dendritic cells are key cellular linkers between innate and adaptive immune responses during fungal infections. For appropriate activation and expansion, in fact, T lymphocytes require an intimate contact (immunological synapse) to DCs, as major antigen-presenting cells (APCs), with fungal peptides/MHC complexes and co-stimulatory molecules on the DC surface mediating effective intercellular communications [152]. These events are of key importance for the generation of antifungal antibodies, in the late stages of an *A. fumigatus* infection. In this regard, raised levels of *Aspergillus*-specific IgG and IgE are often found in individuals with chronic pulmonary aspergillosis and allergic aspergillosis, respectively. Indeed, antibody levels are used in the clinic to monitor treatment response in these syndromes, however antibody-based tests often have critical limitations in specificity and reproducibility [153].

Different studies on the role of T helper (Th) cells in *A. fumigatus* infections point to dysregulation of the Th1/Th2 balance and its bias towards Th2 immune responses as key cellular and molecular events in susceptible humans and mice [154-156]. DCs are fundamental to orient polarization of T cells, therefore directly impacting on the Th1/Th2 balance. The complexity of this process in the course of an *Aspergillus* infection clearly emerged in a study by Bonifazi *et al.* [157], where using *Aspergillus*-pulsed and adoptively

transferred lung- or bone marrow-derived DCs, fungal conidia were described to initiate more protective Th1/regulatory T cell (Treg) responses, whereas hyphae were mostly found to trigger inflammatory Th2/Th17 reactions. These findings indicate that sensing of different fungal morphotypes by DCs activate distinct signaling pathways and intercellular networks. Furthermore, given that Tregs play a crucial role in the control of immune reactions and dampening of the inflammatory response, DCs are strategic to prevent autoimmune and autoinflammatory damage to the healthy host tissue [158-160].

1.3.1.4 THE JOURNEY OF *ASPERGILLUS FUMIGATUS* ACROSS INNATE IMMUNE CELLS

The different cell types of innate immunity cooperate to, recognize, phagocytose and kill *A. fumigatus*. They also make a “bridge” for initiation and orientation of the adaptive response.

As described above, the first encounter of *A. fumigatus* airborne conidia with the host innate immune system involves lung-resident alveolar macrophages. At the beginning of an infection, the fungal spores can be recognized by DC-SIGN (and other receptors) on these cells, and are eventually phagocytosed and killed. When required, alveolar macrophages can mount a pro-inflammatory reaction with activation and recruitment of other cell types to the site of infection.

Lung DCs are fundamental for initiation of an appropriate adaptive immune response. Indeed they guide polarization of the T cell compartment, regulating the Th1/Th2 balance, a process that is affected by the host immune status and the encountered fungal morphotypes. As illustrated above, resting conidia preferentially initiate Th1/Treg responses, whereas hyphae mostly trigger Th2/Th17 reactions.

Although recruited within a few hours from infection, neutrophils are the master orchestrators of the host reaction to *Aspergillus fumigatus* infections, as indicated by a large body of both pre-clinical and clinical evidence (see section 1.3.1.1). Neutrophil-mediated killing of *A. fumigatus* involves a number of mechanisms, including production of ROS, formation of NETs, release of microbicidal components from intracellular granules, and phagocytosis of the fungal particles [111]. Defects either in number, recruitment or function of PMNs are long known to be associated with increased

susceptibility to IA, which further highlights the importance of these cells in the containment of *A. fumigatus* infections.

Furthermore, of relevance to the scopes of this study, here I anticipate that genetic deficiency of PTX3 (a long pentraxin that is stored as a pre-made protein in the specific granules of human neutrophils) impairs the antifungal effector mechanisms of these cells, namely phagocytosis and killing [161]. The specific impact of PTX3 deficiency on neutrophil function has been recently corroborated by the loss of the genetic association in patients that developed IA during severe neutropenia [162].

1.3.2 THE HUMORAL ARM

The humoral arm of the innate immune system comprises a number of germ-line encoded soluble PRMs that differ in structure, cellular source, inducing stimuli and mechanisms of action [163]. In this regard, neutrophil granules are a reservoir of humoral PRMs (e.g., PTX3, M-ficolin, and complement proteins) to be rapidly released within minutes from infection [163]. Early gene expression-dependent production by mononuclear phagocytes and DCs sustains levels of a wider repertoire of soluble molecules [164]. Finally, epithelial tissues (the liver in particular) act as a source of delayed, systemic PRMs [165]. Not only they are produced in response to microbes or tissue damage, but some PRMs are also present constitutively to provide a ready to use “stock” in the circulation or in the tissue (e.g., in the lung) [98]. Humoral PRMs have opsonic activity, and facilitate recognition of pathogens by phagocytes directly or via complement activation [166]. Therefore, despite the impressive structural diversity across PRMs, the general mode of action of these humoral effectors is remarkably similar to that of antibodies (“ante-antibodies”) [167]. Furthermore, a close functional cooperation exists between PRMs from different families, which results into formation of dynamic multi-molecular complexes with distinct functional properties [163]. Such complexity extends to the cellular arm of the innate immune system, whose components not only act as a source of humoral PRMs, but are also targets of these soluble effectors, which shape and optimize their function (e.g., in the phagocytosis of opsonised pathogens) [7]. Therefore, the humoral and cellular components of innate immunity form an integrated system with crosstalk, synergism and regulation (see Figure 4 for an overview of PAMPs and PRRs/PRMs involved in the immune response to *A. fumigatus*) [7]. Major soluble players of the innate immune system are

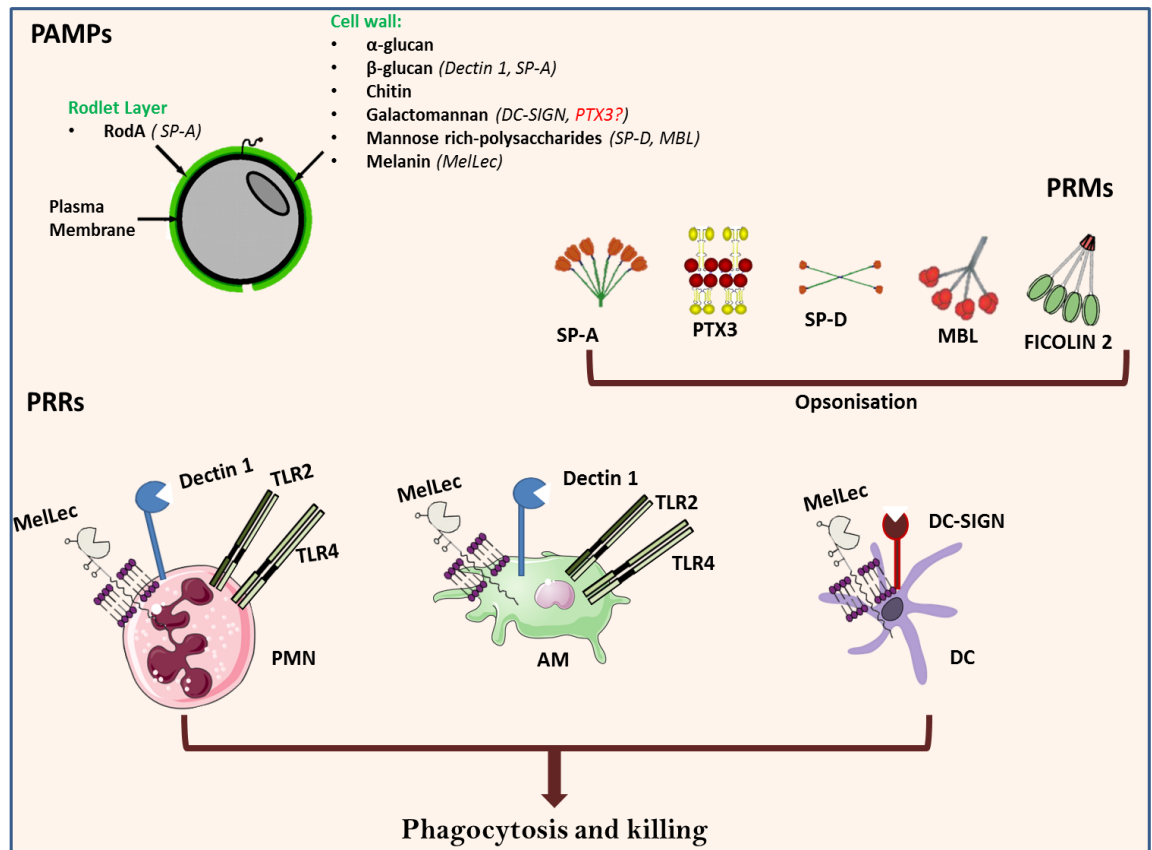


FIGURE 4. PAMPs and PRRs/PRMs involved in the immune response to AF. The major PAMPs expressed by *A. fumigatus* and their corresponding host-derived PRRs/PRMs (italics in brackets) are indicated (upper left panel). Inhaled resting conidia are engaged by soluble PRMs (i.e., MBL, PTX3, SP-A/D, ficolin-2) that act as opsonins (middle panel). They are also recognized by cell-associated PRRs, like MelLec (that binds melanin) and DC-SIGN (that senses galactomannans) (lower panel). Conidial swelling can occur in the bronchoalveolar space or within alveolar macrophage phagolysosomes and results in β -glucan surface exposure, triggering inflammatory and fungicidal responses through the mammalian β -glucan receptor, dectin-1. TLR2- and TLR4-dependent signals contribute to host recognition of germinating conidia and hyphae, as well. Recruited neutrophils cooperate to inactivate conidia and, together with alveolar macrophages and dendritic cells, prevent conidial germination in immune-competent hosts. Adapted from Heinekamp et al [3].

complement, collectins, ficolins and pentraxins [168-170]. Collectins and ficolins will be shortly introduced in the following chapters, whereas separate sections will be dedicated to the complement system and pentraxins.

1.3.2.1 COLLECTINS

Collectins (also known as collagen domain containing C-type lectins) are soluble PRMs that recognize the sugar moieties present on pathogens, including the cell wall of *A.*

fumigatus, and share common structural features, including a triple-helical collagen region and a carbohydrate recognition domain (CRD) [171]. The pulmonary collectins surfactant proteins A and D (SP-A and SP-D, respectively) bind glucan- and mannose-rich polysaccharides of the fungal cell wall [172]; also, SP-A recognizes two N-glycosylated glycoprotein antigens that are secreted by *A. fumigatus* (i.e., gp45 and gp55) [173]. The interaction of both collectins with *A. fumigatus* conidia increases phagocytosis and fungal killing by neutrophils and alveolar macrophages, via a calcium-dependent yet unknown receptor [171]. An immunomodulatory role of lung collectins in the host defense from *A. fumigatus* has been reported both in mice and humans [142,173]. SP-D gene-deficient mice are more susceptible to invasive pulmonary aspergillosis than their wild type littermates, and intranasal administration of exogenous SP-D has been shown to protect immunosuppressed mice in a murine model of invasive pulmonary aspergillosis [174]. However, SP-A gene-deficient mice are resistant to the disease, suggesting that SP-A may be involved in its pathogenesis [175,173]. Furthermore, patients with *Aspergillus* infections and allergies have alterations in their serum and lung lavage levels of SP-A and SP-D, and epidemiological studies have found associations between polymorphisms in the SP-A and SP-D genes, levels of the corresponding proteins and susceptibility to fungal diseases [176,177,174].

Another member of the collectin family that shares close structural similarity with SP-A and SP-D, is the serum protein mannan-binding lectin (MBL) [178]. In addition to binding and opsonizing properties, MBL also activates the lectin pathway of complement (LP, see section 1.4) via MBL-associated serine proteases (MASPs) and induces the production of pro-inflammatory cytokines [179]. Single nucleotide polymorphisms (SNPs) in exon 1 (which encodes the collagen region of the protein) and the promoter region of the gene have been associated with reduced serum levels of MBL and increased susceptibility to a number of pulmonary infections, including tuberculosis, cystic fibrosis and pulmonary aspergillosis, thus suggesting an important role for MBL in pulmonary host defense [180]. MBL binds *A. fumigatus* conidia *in vitro* [178], and is the main complement activator and phagocytosis initiator on these fungal spores in individuals with reduced levels of anti-*Aspergillus* IgG [181]. Despite being a prototypical initiator of the LP, MBL has been proposed to activate the alternative pathway (AP) of complement when bound to *A. fumigatus* conidia via a C2 bypass mechanism that does not require cleavage of C4 and C2 (see section 1.4).

1.3.2.2 FICOLINS

Ficolins are a family of soluble PRMs that share with collectins a similar structural organization (a collagenous region linked to a recognition domain), however in the place of CRD they contain a fibrinogen-like domain (FD) [182]. The family comprises three members, namely ficolin-1 (or M-ficolin), ficolin-2 (or L-ficolin) and ficolin-3 (or H-ficolin), which differ in function and site of synthesis. Ficolin-1 is predominantly expressed in peripheral blood leukocytes, ficolin-2 is mainly produced in the liver, and Ficolin-3 is synthesized both in the liver and lung [183]. From a functional perspective, the three ficolins recognize a wide spectrum of microorganisms via their FD region and, similar to MBL, activate the LP through association of their collagen-like domain with MASPs [184]. Ficolin-2 preferentially binds to β -1,3-glucans and N-Acetylglucosamine-rich polysaccharides in the cell wall of *A. fumigatus*, and triggers activation of the LP via MASP-2 *in vitro* [185]. In agreement with this, ficolin-2 has been found in the bronchoalveolar lavage fluid from the lungs of patients with IA [186]. Furthermore, genetic deficiency of ficolin-A (the mouse paralog of human ficolin-2) has been reported to increase the fungal load in an animal model of IA [187]. The murine protein recognizes *A. fumigatus* conidia, however, in contrast to the human counterpart, it does not activate the LP, but rather enhances adherence of the fungal spores to a lung epithelial cell line (i.e., A549), and modulates inflammation via production of pro-inflammatory cytokines *in vitro*. Therefore, it is likely that this ficolin elicits species-specific effector mechanisms in humans and mice [188]. Within the general frame of a functional cooperation between different PRMs (as described above), ficolin-2 interacts with the short pentraxin C reactive protein (CRP, see section 1.5), stabilizing its interaction with selected bacteria [189]. This collaboration has been shown to enhance the recognition of *S. enterica* and activate the LP on the surface of this pathogen [189]. This concept has been extended to opportunistic pathogens, like *Pseudomonas aeruginosa* [190], *Candida albicans* [191] and, importantly, *Aspergillus fumigatus* [86]. Furthermore, ficolin-2 has been shown to cooperate with another pentraxin, PTX3 (see section 1.6), in the immune response to *A. fumigatus*. In this regard, formation of ficolin-2/PTX3 hetero-complexes has been reported on the conidial surface, which boosts complement activation and fungal disposal, an additional example of PRM crosstalks that expand and amplify the pattern recognition repertoire of the host [86].

1.4 THE COMPLEMENT SYSTEM

The complement system comprises a large number of both soluble and membrane-associated proteins that coordinately mediate several effector functions, including recognition of pathogens and/or damaged host cells, and their elimination via either direct (i.e., formation of the membrane attack complex, MAC; see section 1.4.4) or indirect (i.e., phagocytosis and phagolysosomal killing) lytic mechanisms [192]. Furthermore, activation of this system elicits a cascade of inflammatory events, including recruitment and activation of immune cells (e.g., PMNs and macrophages), which help fight and restrain infections [193]. Furthermore, complement has an active role in the regulation of the antibody response, and in the clearance of immune complexes and apoptotic cells [194,195]. However, if left unchecked, complement activation might lead to host tissue damage and anaphylaxis. Indeed, major disorders associated to complement deficiencies (mostly those involving complement regulators) are recurrent severe infections as well as autoimmune and autoinflammatory diseases [196]. The importance of complement is also emphasized by its evolutionary conservation in as distant species as mammals, birds, reptiles, amphibians, and ascidians [197].

Initiation and activation of the complement system involves a cascade of proteolytic events in a feed forward network of active enzymes and zymogens, which closely resembles that of the coagulation system [198]. In this cascade, the inactive complement zymogens are successively cleaved to yield two fragments, the larger of which is an active serine protease, and the smaller is released from the reaction site acting, in some cases, as a soluble inflammatory mediator [199].

There are three distinct pathways through which the complement system can be activated: classical, lectin and alternative (CP, LP and AP, respectively; see Figure 5) [200,201]. These are initiated by different molecules and mechanisms, however they converge downstream in the cascade to generate overlapping sets of effector molecules [202]. The point of convergence is represented by the AP C3 convertase, where the AP can act as both an independent pathway and an amplification loop for CP and LP.

This convertase (i.e., a proteolytically active C3b/Bb complex) catalyzes the breakdown of C3 (the “core” and most abundant component of the system) into C3b, which can form covalent bonds with nucleophilic groups on activator surfaces (e.g., *non-self* pathogens or damaged/altered *self* tissues) via a highly reactive thioester function, and

C3a, an anaphylatoxin that acts as a soluble mediator of inflammation. The newly generated C3b is *per se* an opsonin that is mostly recognized by complement receptor 1, CR1 (or CD35), abundantly expressed on several phagocytes, including PMNs; furthermore, it participates to the assembly of additional convertase complexes, thus amplifying complement activation and promoting its downstream pathways, which leads to

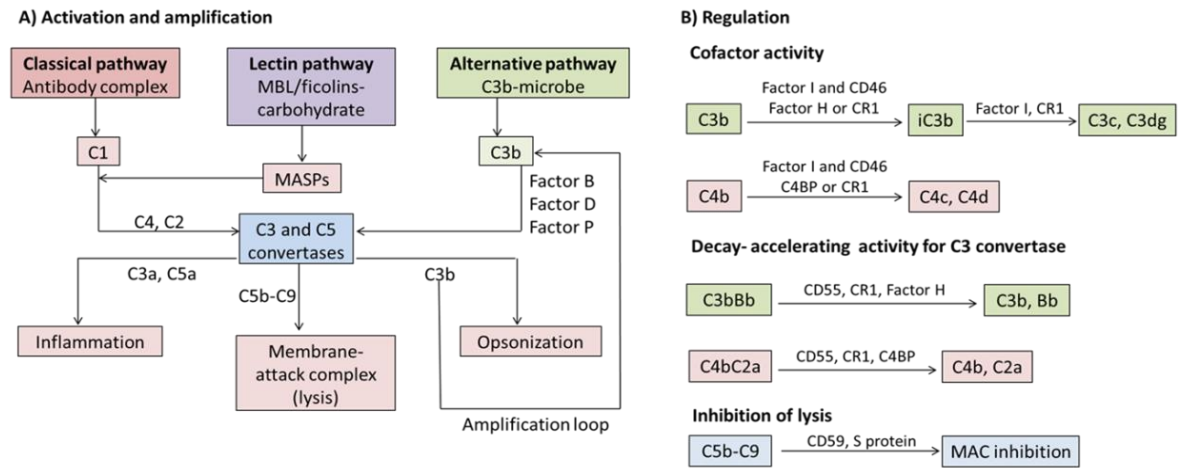


FIGURE 5. The complement system. **A)** Activation of classical (CP) and lectin (LP) pathways is restricted to recognition of immune complexes and non-self carbohydrates by soluble receptors (i.e., the C1q moiety of C1, mannose-binding lectin (MBL) and ficolins). Associated serine proteases (i.e., C1r/C1s for C1, MASP2s for MBL/ficolins) then cleave C4 and C2, and generate C4b and C2a fragments that form the CP/LP C3 convertase (C4bC2a). This complex catalyzes conversion of C3 into C3a and C3b, where the latter contains a highly reactive thioester that forms covalent bonds with proximal nucleophiles. Cell-bound C3b can engage in a complex with factor Bb (i.e., produced by the factor D-dependent cleavage of factor B, FB) and form the alternative pathway (AP) C3 convertase (C3bBb), which is stabilized by factor P (properdin) and produces additional C3b. Therefore, AP can act as an amplification loop for both CP and LP. However, AP activation can occur in the absence of a recognition event; indeed, C3 undergoes hydrolysis (i.e., C3(H₂O)), and form a convertase with factor Bb after cleavage of FB by factor D, (C3(H₂O)Bb) in solution; this cleaves C3 to C3b, where the latter can become immobilized on a surface (again via thiol ester). If not inactivated the cell-bound C3b can then form the AP convertase and amplify complement deposition. All three pathways converge into formation of C3 and C5 convertases that, in turn, generate the anaphylatoxins C3a and C5a (chemoattractants and activators of immune cells), the membrane-attack complex (MAC, C5b-C9, which causes lysis of the target cells) and C3b (that opsonises pathogens and promotes their phagocytosis). **B)** Self tissues are protected from unwanted complement deposition by fluid-phase and cell-associated regulators. C3b and C4b undergo proteolytic processing by factor I (FI). This enzyme requires cofactors, which are either membrane proteins (CD46 and complement receptor 1, CR1) or fluid-phase regulators (factor H (FH) and C4-binding protein (C4BP), respectively). The dissociation rate of C3 and C5 convertases (the so-called decay-accelerating activity) is enhanced by CD55, CR1, FH and C4BP. Formation of MAC is controlled by CD59 and S protein (vitronectin). Adapted from *Kemper and Atkinson* [8].

disruption of the target cell (e.g., via formation of MAC that inserts into the cell membrane

and causes cell lysis or apoptosis) and enhanced inflammation (i.e., via production of the other anaphylatoxin C5a) [199]. In the case of binding to non-activator surfaces (e.g., healthy *self* tissues), C3b is proteolytically inactivated to iC3b (and smaller fragments, like C3dg and C3d) by a soluble serine protease (i.e., FI) in association with both fluid phase (i.e., FH) and membrane-bound (i.e., membrane cofactor protein (MCP), also known as CD46, and CR1) co-factors. These mechanisms are important for tissue homeostasis, since they support effective removal of cellular debris (e.g., following apoptosis) by professional phagocytes (where iC3b is a major opsonin recognized by complement receptors 3 (CR3), also termed CD11b/CD18, and 4 (CR4), also known as CD11c/CD18) and contribute to dampen/restrain inflammation [203].

Thus, the complement system is endowed with several effector functions, which can be summarized as follows: *i*) opsonisation of target cells and promotion of their phagocytosis by immune cells, *ii*) formation of MAC, which leads to cell death through apoptosis or mechanical disruption, *iii*) inflammation, which is enhanced by the anaphylatoxins C3a and C5a that act as chemotactic (i.e., by promoting the migration of phagocytes to the site of complement activation), pro-inflammatory (i.e., via activation of both immune and somatic cells and induction of inflammatory cytokines), and vasoactive agents (i.e., by stimulating the release of vasoactive molecules from mast cells and basophiles). Finally, complement can link innate and adaptive immunity [204]. In fact, not only antigen/antibody complexes are recognized by the CP (see below), but also B cell differentiation, long term memory, and transfer of complement-tagged antigens to lymph nodes and follicular dendritic cells all involve an active participation of the complement system [205].

1.4.1 CLASSICAL PATHWAY

The classical pathway is triggered by activation of the C1-complex (a single C1q molecule, which acts as a recognition unit, and two each of the serine proteases C1r and C1s), which occurs when C1q binds to IgM or IgG complexed to antigens (i.e., Ig-dependent activation) [206], or when C1q binds the activator surface either directly (e.g., via recognition of chromatin and cardiolipin) or indirectly (e.g., via adaptor proteins like pentraxins, see below) (i.e., Ig-independent initiation) [207,208]. Such binding events lead to conformational changes in the C1q molecule, which results into activation of C1r and the

subsequent C1r-mediated cleavage of C1s [202]. The active C1 complex binds to and cleaves first C4 then C2, producing C4a, C4b, C2a and C2b. C4b and C2a can form the CP C3 convertase (C4b2a), which catalyzes cleavage of C3 into C3a and C3b, and acts as a scaffold for generation of the CP C5 convertase (C4b2a3b), which splits C5 into C5b and C5a, where C5b proceeds to MAC formation [192]. C1q has a hexameric structure that resembles a bouquet of tulips in electron microscopy [209]. It comprises an N-terminal collagen-like region, which forms triple helices with fibril-like organization, and is connected to a C-terminal globular domain (gC1q). The gC1q domain has a heterotrimeric organization, formed by the C-terminal domain of 3 (A, B, C) different chains, which makes up to 6 globular heads [210]. Unlike most complement proteins which are produced in the liver, C1q is predominantly synthesized by tissue macrophages, dendritic cells and endothelial cells, and localizes in the extracellular matrix [211]. Only a fraction of C1q found in circulation forms the C1 complex; furthermore, activation of this complex is tightly controlled by inhibitors, most importantly C1-inhibitor [212].

1.4.2 LECTIN PATHWAY

The lectin pathway shares with the CP similar activation mechanisms and common effector components (i.e., C4 and C2), however it uses MBL and ficolins in the place of C1q as initiator units [202]. MBL specifically binds to mannose residues (and, to a lesser extent, other sugar moieties), and is able to initiate complement activation on surfaces rich in these structures (i.e., microbes and damaged host endothelium) [213,214]. MBL, like C1q, is a six-headed molecule that forms a complex with two protease zymogens, MASP-1 and MASP-2. These are close homologous of C1r and C1s, and all four enzymes likely evolved from a common ancestor via gene duplication [215]. Following binding of MBL to the activator surface, MASP-1 and MASP-2 acquire the ability to cleave C4 to C4a and C4b, and C2 to C2a and C2b, similar to C1r and C1s [216]. C4b and C2a can then form the CP/LP C3 convertase, as described for the classical pathway [217]. Similarly to MBL, the three human ficolins associate with MASP-1, MASP-2 and a truncated form of MASP-2 called small MBL-associated protein (sMAP) [218], and activate the LP, via cleavage of C4 and C2 and formation of the CP/LP C3 convertase. In this regard, ficolins have been reported to opsonise selected microbes and promote their phagocytosis. For example, complexes formed by L-ficolin and MASPs bind to intact group B streptococci and promote C4 consumption [219]. In addition, ficolins interact with CRP, thus amplifying

the complement-mediated killing of *P. aeruginosa* and widening the range of bacteria recognized by CRP [189] (see section 1.5.2).

1.4.3 ALTERNATIVE PATHWAY

The third pathway of complement is called alternative because it was discovered as a second, or 'alternative', mechanism of activation after description of the classical one [220]. In contrast to the classical and lectin pathways, the AP does not depend on a recognition event for its initiation; instead, it is continuously active at a low rate in the plasma and certain tissues (e.g., in the eye) [200] through the spontaneous hydrolysis of C3, also known as C3 'tick-over' [200]. In this regard, full activation of the AP requires four plasma proteins: C3, factor B (FB), factor D (FD) and properdin (or factor P, FP). C3 (an abundant protein in the human plasma, with an average concentration of 1.2 mg/ml) contains a highly reactive thioester that undergoes hydrolysis to form C3(H₂O), a form of C3 that can bind FB. When in a complex with C3(H₂O), FB becomes a substrate for the serine protease FD, which cleaves FB to Ba and Bb, the latter remaining associated with C3(H₂O) to form the C3(H₂O)Bb complex. This complex is the initial C3 convertase of the AP that can cleave fluid phase C3 molecules, generating metastable C3b fragments (with an half-life of ~60 μ s) that retain the thioester function originally present in the parent C3 [202]. Within a short time the newly formed C3b can attach covalently to nucleophiles (hydroxyl and amino groups) on proximal surfaces or water molecules. C3b bound in this way is able to associate with FB, thus forming the C3 pro-convertase of the AP (C3bB). This is then converted to the AP C3 convertase (C3bBb) upon FD-mediated hydrolysis of B to Bb. The C3bBb complex has a short half-life (2-3 min at 37°C), which however is extended by 10-fold by properdin [221], and acts as an enzyme that cleaves and activates other C3 molecules. Subsequently, a positive feedback loop is created, which leads to the increased C3b deposition on the nearby surface through formation of new C3 convertases and C3b molecules. C3bBb also acts as a molecular scaffold for assembly of the C5 convertase of the AP (C3bBbC3b), which extends the activation process to the terminal pathway (see below) [200] (see Figure 3).

1.4.4 *TERMINAL PATHWAY*

The terminal pathway of complement consists of five plasma proteins (C5, C6, C7, C8 and C9), and is initiated when C5 convertases of the CP/LP and AP cleave C5 into C5a and C5b [192]. It is worth mentioning here that transition of the C3 convertases of all pathways to the their C5 forms (i.e., that differ in composition from the corresponding C3 convertases for the presence of one C3b molecule only) depends on the cell surface density of C3b. In this respect, low densities of C3b favour formation and activity of C3 convertases, with increased opsonisation (and eventually phagocytosis) of target cells as a prominent functional outcome; on the contrary, when the C3b/cell ratio is high, C5 convertases are favoured, which promotes the terminal pathway and cell lysis [192,222]. In the latter conditions, the newly formed C5b binds C6, and this interaction induces a conformational change in C6 that makes it capable to recognize C7 [192]. After addition to the C5bC6 complex, C7 acquires binding to the lipid bilayer of the plasma membrane. This binding ability is short-lived, therefore if the C5b-7 complex does not attach to the plasma membrane within a short while, plasma S-protein (vitronectin) binds to it leading to formation of a lytically inactive SC5b-7 complex. Plasma C8 recognize both membrane-bound C5b-7 and fluid-phase SC5b-7 complexes through its β chain [223]. The membrane bound C5b-8 complex then acts as an initiator of the membrane attack complex (MAC), which forms large, 10 nm wide, pores in the target membrane [224]. For functional pores to form, at least one C9 molecule is required to bind the C5b-8 complex. However, a single MAC can contain up to 18 C9 molecules that polymerize and form a tubular channel, highly resistant to environmental changes (e.g., denaturing agents and detergents) [225,192]. The subsequent alterations in membrane permeability and mechanics lead to irreversible osmotic damage of the target cell and eventually lysis. A soluble counterpart of the MAC is also formed based on SC5b-7 that comprises SC5b-9 complexes, known as terminal complement complexes (TCC) [226-228].

1.4.5 *SELF VERSUS NON-SELF DISCRIMINATION: HOW THE COMPLEMENT SYSTEM IS REGULATED*

Discrimination between pathogenic invaders and the host's own cells is critical for host survival, as are means of killing or expelling the invading microorganisms. There is evidence of complement-like components in primitive coral and sponges (early

multicellular organisms), suggesting that the discriminatory mechanisms of this system have had approximately a billion years to develop [229,230]. Each of the three complement pathways uses its own unique mechanisms for target versus host discrimination [202]. The alternative pathway primarily uses reverse recognition: it detects structures/molecules on the host cell that inhibits it (markers of *self*) and become activated on anything that lacks such markers (*non-self*) [202]. Factor H (FH) is a paradigmatic example of reverse recognition, in that it recognizes host-associated molecular patterns (HAMPs), thought to be primarily surface polyanionic structures, and inhibits complement activation on host surfaces, thus acting as an host pattern recognition molecule (HPRM) [231-235]. As opposed to this, activation of both the LP and CP is triggered by direct recognition of *non-self* PAMPs, via initiator molecules that act as soluble PRRs [8]. In this regard, the lectin pathway uses members of the collectin and ficolin families of proteins to recognize PAMPs, such as polysaccharides, on microbial surfaces (see “Collectins” and “Ficolins”). The specificities of the lectin sites on these proteins have only begun to be characterized. The classical pathway is initiated by the binding of C1q to antigen-antibody complexes, certain pentraxins, as well as some PAMPs. This complement pathway primarily depends upon the very sophisticated discriminatory processes of the adaptive immune system to mount responses to pathogens and eliminate responses to autoantigens [202]. Furthermore, direct pathogen recognition by an alternative pathway protein has been described. Properdin was indeed found to bind to and activate the alternative pathway on *Neisseria* [236].

Clearly any errors in target identification that result in complement activation on host cells or tissues can have devastating consequences to the host; therefore, activation of the complement system has to be tightly regulated. Currently, five membrane-bound and six soluble regulators are known. Complement receptor type 1 (CR1, CD35), membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55) and CrIg (complement receptor of the immunoglobulin superfamily) inhibit complement activation at the C3 convertase level [202], whereas protectin (CD59) inhibits the formation of MAC. Amongst the soluble regulators, FH and factor H-like protein 1 (FHL-1), an alternatively spliced product of the FH gene, regulate the amplification loop of the AP [200], whereas C1-inhibitor and C4b binding protein (C4BP) inhibit the CP/LP [237]. In addition, clusterin and vitronectin act on the terminal pathway [226].

1.4.6 COMPLEMENT AND *ASPERGILLUS FUMIGATUS*: A HIDE AND SEEK GAME

A. fumigatus, both in the form of conidia or hyphae can activate the three complement pathways [238]. In particular, the CP and LP play predominant roles in the complement response to conidia, with the AP contributing as an amplification loop [181]. In this regard, MBL is a major soluble PRM both in recognition and disposal of *A. fumigatus* germlings/hyphae via activation of the LP (see “Collectins”) [181].

A. fumigatus has evolved several strategies in order to escape the complement-mediated immune response:

- *Hiding*. Aspergillosis is often characterized by the formation of abscesses, mainly if the central nervous system is involved [239]. Even in the course of an active inflammatory reaction, conidia can swell and invade the parenchymal tissue of the brain, white blood cells are recruited, and a fibrous tissue (capsule) forms around the infected area. This capsule acts as a host-defense mechanism to avoid dissemination of the invading pathogen. However, it also prevents access of immune effector molecules (e.g., complement) to the site of infection, thus hiding the fungal pathogen to complement attack [240];
- *Masking*. PAMPs present on the conidial surface of *A. fumigatus* are masked to complement recognition. A major role in this regard is played by RodA, whose hydrophobic rodlets mask the polysaccharides present in the fungal cell wall not only to phagocytic receptors (e.g., dectin-1) but also to MBL and ficolins (see above and [83]). Despite fungal melanin being recognized by the MelLec receptor [69], this cell wall component might provide an additional masking strategy [241]. In this regard, it has been reported that genetic deletion in *A. fumigatus* of a key enzyme in the biosynthetic pathway for melanin results in increased opsonisation of fungal conidia by C3 and augmented phagocytosis of the pathogen by professional phagocytes [242]. However, even though fungal melanin has been implicated in the scavenging of ROS and inhibition of the phagolysosome acidification (see above), how it protects this fungus from complement attack is still unknown [243-245];
- *Recruitment of complement inhibitors*. *A. fumigatus* has been shown to hijack soluble inhibitors of both the AP and CP/LP, including FH, FHL-1, and C4BP [82,246]. In particular, when bound to the conidial surface, FH retains the ability to act as a cofactor for the factor I-mediated cleavage of C3b, therefore inhibiting activation and amplification of the complement response on this fungal pathogen;

- *Production of complement inhibitors.* *A. fumigatus* is not only able to recruit complement inhibitors, but it also produces its own soluble factors that inhibit complement activation [247]. In particular, these molecules target the alternative pathway and prevent conidia opsonisation by C3 and its fragments. The identity of this class of microbial complement inhibitors is still unknown, although available evidence suggests that they might be polysaccharides and/or protein/glycan conjugates [248];

- *Degradation of complement proteins.* *A. fumigatus* is able to synthesize and release proteases (e.g., Alp1) that target and degrade key complement proteins, including C1q, C3, C4 and C5, thus providing an additional mechanism to inhibit or even block the generation of antimicrobial effector molecules [249-251].

In addition to direct antifungal activities of complement, a close crosstalk in the immune response to *A. fumigatus* has been described between this system and other soluble PRMs, with major regard to the long pentraxin PTX3, which will be discussed in the following sections [181,2].

1.5 PENTRAXINS

Pentraxins are a family of multimeric pattern recognition proteins highly conserved in evolution [252]. These are characterized by the presence in their carboxy-terminus of a 200 amino acid pentraxin domain, with an 8 amino acid long conserved pentraxin signature (HxCxS/TWxS, where x is any amino acid) [170]. The pentraxin domain has also been found in multidomain proteins, such as the extracellular protein Polydom/Svep1 [253] and a few adhesion-G-protein coupled receptors, in particular GPR144 [254]. However, the role of the pentraxin domain in these molecules has not been defined as yet. Based on the primary structure of the protomer, pentraxins are divided into two groups: short pentraxins and long pentraxins [166].

1.5.1 SHORT PENTRAXINS

Short pentraxins are ~25 kDa proteins characterized by a common structural organization, with five or ten identical subunits arranged in a pentameric radial symmetry [166]. Human C reactive protein (CRP) and serum amyloid P component (SAP) are the prototypic short

pentraxins: they share approximately 51% amino acid sequence identity and are thought to originate from a single gene duplication [255]. Both CRP and SAP have been found in all vertebrates which they have been sought in [256,257]. CRP is also present in the hemolymph of invertebrates, such as the arthropod *Limulus polyphemus* and the mollusk *Achatina fulica* [258,259]. CRP was the first pentraxin and soluble PRM to be identified in human serum in the 1930s as an acute phase response protein [260]. Human SAP was subsequently described as a relative of CRP on the basis of amino acid sequence similarity and a similar appearance in electron microscopy (annular disc-like structure with pentameric symmetry) [261]. CRP and SAP orthologs in different mammalian species share substantial sequence similarity, with notable differences in basal serum levels and changes during the acute phase response. In this regard, although the pattern-recognition properties of these short pentraxins towards a wide range of ligands have been conserved throughout evolution, their acute phase nature is species-specific. For example, CRP and SAP are the main acute phase reactants in human and mouse, respectively, whereas in rats CRP is constitutively expressed at relatively high levels, and these change only marginally in the course of an acute phase reaction [262].

The human CRP protein is composed of five identical non-glycosylated protomers, containing two cysteine residues at positions 36 and 97 that are highly conserved in all members of the pentraxin family and involved in intra chain disulfide bonds [263]. Each CRP protomer has a characteristic Concanavalin-A like lectin fold composed of two-layered β sheets with flattened jellyroll topology; five protomers are non-covalently associated to form a pentamer with a total molecular weight of ~115 kDa [263]. The human CRP gene is located on chromosome 1q23 and is organized in two exons, the first coding for the signal peptide and the initial two amino acids of the mature protein and the second coding for the remaining 204 amino acids. The promoter region of human CRP comprises two acute phase response elements, each containing a binding site for the liver-specific transcription factor HNF1 [264], and two C/EBP (CCAAT/enhancer binding protein β) binding sites, both necessary and sufficient for IL-6-induced transcription [265]. CRP levels in the plasma of healthy adults are detectable (≤ 3 mg/l) but increase as much as 1000-fold following an acute phase stimulus as a result of accelerated rates of transcription in the liver [166]. Indeed, circulating CRP is produced mostly by hepatocytes, mainly in response to the pro-inflammatory cytokine IL-6. IL-1 may also contribute as an additional signal that synergizes with IL-6 in inducing the CRP mRNA [266]. CRP has been used

extensively in the clinic for over 75 years as a nonspecific systemic marker of infection, inflammation, and tissue damage [7].

SAP is a highly conserved plasma glycoprotein composed of 5 or 10 identical subunits non-covalently associated in pentameric rings interacting face to face [261]. The human gene maps in chromosome 1 in close physical and genetic linkage with the CRP gene and shares with CRP the same organization in two exons, the first one coding for the signal peptide and the second for the mature protein. The mature SAP protomer is 204 amino acid long and has a molecular mass of ~25 kDa. Unlike CRP, each SAP protomer is glycosylated with a single N-linked biantennary oligosaccharide at Asn32 [267]. According to the three-dimensional crystal structure, the five composing subunits of human SAP are arranged like a ring around a hole and are held together by hydrogen bonds and salt bridges [267]. Two pentamers are stacked in a decameric arrangement that is stabilized by additional ionic interactions. Each SAP subunit can bind two calcium ions, and residues involved in calcium binding are conserved in all species. SAP is produced exclusively by hepatocytes and is the main acute phase protein in mice, whereas in the human serum it is constitutively present at 30 to 50 mg/l [7].

1.5.2 FUNCTION OF THE SHORT PENTRAXINS

The physiological functions attributed to pentraxins involve recognition and binding to different ligands, mostly in a calcium dependent manner [268]. In this regard, the first ligand described for CRP was the C-polysaccharide of *Streptococcus pneumoniae*: this interaction is due to a direct binding of CRP to phosphorylcholine (PC), a major constituent of the C-type capsule polysaccharides [269]. Moreover, CRP binds various microbes, including fungi, yeasts, bacteria and parasites through PC and carbohydrate structures, promoting phagocytosis and resistance to infections [270]. Binding to bacteria is not always necessary for protection since CRP also protects mice from infection with *Salmonella typhimurium*, a pathogen to which CRP does not bind [271]. Furthermore, CRP-mediated activation of the classical complement pathway (see below) has no role in protecting mice against *S. pneumoniae* infection [272]. Like CRP, SAP binds various bacteria, such as *Streptococcus pyogenes* and *Neisseria meningitidis* [273]. Moreover, binding to influenza virus has also been reported [274]. SAP binds to several bacteria via lipopolysaccharide (LPS) and prevents LPS-mediated complement activation and LPS

toxicity [275]. However, for certain microorganisms that are recognized by this short pentraxin, such as *S. pyogenes* and rough strains of *E. coli*, SAP enhances virulence by protecting the bacteria from phagocytosis, whereas it is host protective in infections mediated by microorganisms to which it does not bind [273].

CRP binds to modified low-density lipoprotein (LDL) via the PC and cholesterol moieties present on LDL [276] and co-localizes with LDL in human atherosclerotic lesions [277]. However, CRP has not been found to be either atheroprotective or proatherogenic in mouse models of human atherosclerosis [278]. Whether increased levels of CRP are in part a cause of cardio-circulatory diseases or are their consequence is currently unclear. Data from both animal and human studies suggest that polymorphisms in the CRP gene, which are linked to marked increases in CRP levels, are not associated with an increased risk of ischemic vascular disease [279,280].

CRP and SAP, either aggregated or when attached to most of their ligands, interact with C1q, the recognition subunit of the CP, and activate the CP cascade [281,282]. Structural studies indicate that CRP binds the globular head of C1q [283]. Complement activation by short pentraxins may also be a mechanism for removal of cellular debris, where complement opsonisation of dead/apoptotic cells prompts their disposal by phagocytes [202]. In this respect, when bound to damaged/altere*d self* surfaces (e.g., apoptotic cells), CRP has been shown to decrease the activity/stability of the AP C3 convertase, thus inhibiting the AP amplification loop, and limiting deposition of C3b (with augmented phagocytosis rather than lysis of the target cells) [189,284]. Inhibition of the AP amplification loop is possibly a consequence of the interaction of CRP with FH [285]. SAP binds to matrix components such as laminin, type IV collagen, fibronectin and proteoglycans [286]. Moreover, SAP binds to the β -amyloid peptide in a calcium dependent manner and contributes to the pathogenesis of amyloidosis [287]. A specific and saturable binding to all three classes of Fc γ receptors (Fc γ R) has been demonstrated for both CRP and SAP and the interaction with Fc γ R is able to mediate phagocytosis of apoptotic cells and microbes [288].

1.6 THE LONG PENTRAXIN PTX3

During the early 1990s, a new pentraxin domain-containing secreted protein was identified as an IL-1 β -inducible gene in endothelial cells and a TNF- α -stimulated gene in fibroblasts,

which was named PTX3 (or TSG-14) [289,290]. This became the prototypic member of the long pentraxin subfamily of proteins that share a common structural organization, with a carboxy-terminal pentraxin-like domain linked to a structurally unrelated N-terminal region. This includes, in addition to PTX3, guinea pig apelin, neuronal pentraxins 1 (NP1, or NPTX1) and 2 (NP2, or NPTX2, also known as Narp), and neuronal pentraxin receptor (NPR), a transmembrane molecule (reviewed in [291]). The amino acid sequence identity among members of this subfamily is relatively high in the pentraxin domain, ranging from 28% between human PTX3 and NP1 to 68% between human NP1 and NP2. By contrast, the amino-terminal domain is poorly conserved (e.g., ~10% identity between human PTX3 and NP1). However, the neuronal pentraxins have a higher degree of sequence identity in their N-terminus (from 28% to 38%), which might suggest the occurrence of a neuronal sub-subfamily among the long pentraxins [291]. Ortholog molecules have been found so far for PTX3, NP1, NP2, and NPR not only in human, mouse, and rat, but also in lower vertebrates such as zebrafish and puffer-fish [292] (Martinez *et al.*, personal communication). Long pentraxins have been identified also in *Xenopus laevis* (XL-PXN1) [293].

1.6.1 GENE EXPRESSION AND PROTEIN STRUCTURE

The *ptx3* gene is remarkably conserved across evolutionary distant species in terms of sequence, structural organization and regulation [166], thus allowing evaluation of its pathophysiological roles in genetically modified animals. In particular, the *ptx3* gene is similarly regulated in humans and mice, indicating that disease modeling in the mouse is likely informative of its functions in humans. As for this, PTX3 expression is rapidly induced by several stimuli, such as inflammatory cytokines (e.g., IL-1 β , TNF- α), TLR agonists, microbial moieties (e.g., LPS, outer membrane protein A, OmpA, lipoarabinomannans), or intact microorganisms in several cell types, including myeloid dendritic cells (DCs), macrophages, endothelial cells, fibroblasts, kidney epithelial cells, synovial cells, chondrocytes, adipocytes, alveolar epithelial cells, glial cells, mesangial cells, granulosa cells, and lymphatic endothelial cells, but not in B and T lymphocytes nor in NK cells [294]. Interestingly, PMNs lack *de novo* synthesis of PTX3; however, they store a constitutive stock of “ready-to-use” protein in specific granules, which is promptly released in response to TLR engagement and localizes in NETs [98]. Expression of PTX3 transcripts (mRNA) is confined to promyelocytes and myelocytes/metamyelocytes (i.e.,

immature myeloid elements), and is missing in bone marrow–segmented and circulating neutrophils (i.e., mature neutrophils). The latter only act as a reservoir of the preformed PTX3 protein. This is found in lactoferrin⁺ and lactoferrin⁺/gelatinase⁺ (specific) granules, with no evidence of protein localization in MPO⁺ (primary or azurophilic) and gelatinase⁺ (tertiary) granules [98].

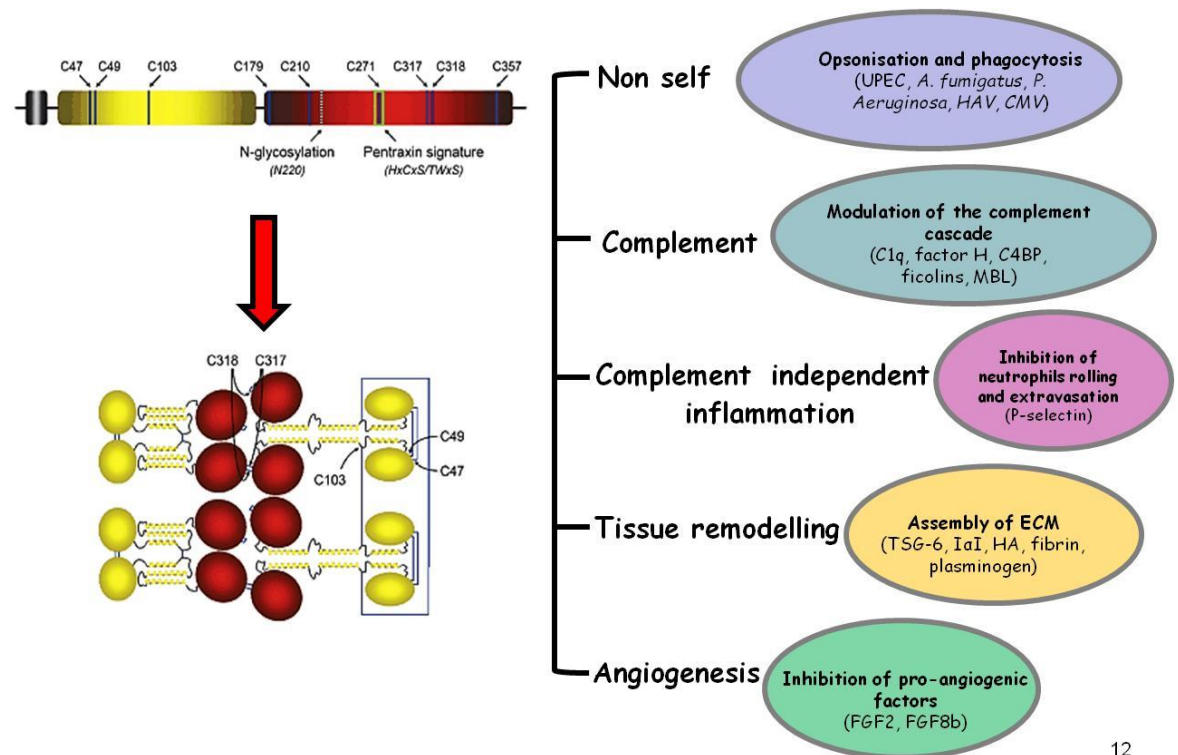
The human PTX3 protomer is a glycoprotein that comprises 381 amino acids, including a 17 amino acid signal peptide, an N-terminal region (18–178), and a C-terminal domain (179–381) [291,289]. A single N-glycosylation site occurs at Asn220 in the C-terminal pentraxin domain that is occupied by complex type oligosaccharides, mainly fucosylated and α -(2,3)-sialylated biantennary sugars [295]. The glycosylation status of the protein (i.e., mainly involving the sialic acid moiety) has been implicated in a number of PTX3 functions in native immunity and inflammation [296] (see below). In addition to the multidomain organization, PTX3 has a complex quaternary structure with eight disulfide-linked protomers folding into an elongated and asymmetric molecule that contains a large and a small domain linked by a stalk region [297,298]. The structural complexity and modular nature of the PTX3 protein likely provide a molecular framework to the rather broad spectrum of cellular and molecular targets of this long pentraxin and the diversity of its biological roles (reviewed in [299]) (Figure 6).

1.6.2 ROLE IN ANTIMICROBIAL IMMUNITY

PTX3 has been recognized as a non-redundant protective factor both in fungal and bacterial infections [141,300]. For example, PTX3-deficient mice are susceptible to invasive aspergillosis [141]. This susceptibility is associated with a low protective Th1

antifungal reaction coupled with an inappropriate Th2 response. Administration of the exogenous protein rescues the Th1 phenotype and has therapeutic efficacy in several models of IA in immunocompromised hosts [141]. Current literature indicates that PTX3 has opsonic activity towards *A. fumigatus*, and enhances recognition, phagocytosis, and killing of fungal conidia by immune cells, mainly PMNs, via complement and Fc receptors (FcRs) pathways [2]. Also, PTX3 has therapeutic activity in animal models of chronic lung infection by *Pseudomonas aeruginosa*, a major cause of morbidity and mortality in cystic fibrosis patients [301]. Also in this condition, the PTX3-dependent recognition and phagocytosis of the pathogen involves a tight interplay with complement and FcRs. Similar

protective roles have been reported for this long pentraxin in the innate resistance to uropathogenic strains of *Escherichia coli* (UPEC), where again it acts as an opsonin by facilitating recognition and phagocytosis of bacteria by immune cells [300]. Furthermore,



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FIGURE 6. PTX3 and its functional roles in innate immunity and inflammation. PTX3 is produced by a number of both somatic and immune cells. The locally released protein is composed of eight identical protomer subunits held together by disulfide bonds, each comprising distinct N- and C-terminal domains (in yellow and red, respectively). The octameric protein folds into an asymmetric molecule with two differently sized domains linked by a short stalk (as exemplified by the schematic drawing of the protein structure). The structural complexity of the PTX3 protein provides a framework for recognition of a multiplicity of ligands, including microorganisms, complement components and modified self-antigens (e.g., apoptotic cells). When bound to microbes and apoptotic cells, PTX3 promotes their disposal by professional phagocytes, via a tight molecular crosstalk with the complement system, and modulates the complement-dependent inflammation. The interaction of PTX3 with viruses can lead to viral neutralization and inactivation (e.g., influenza virus A, (HA), and cytomegalovirus (CMV)). PTX3 exerts anti-inflammatory roles in a complement-independent fashion, for example by reducing leukocyte recruitment to inflamed sites through a specific interaction with P-selectin. As a component of the extracellular matrix (ECM), PTX3 participates in remodeling of hyaluronan (HA) and fibrin networks, at sites of inflammation and tissue injury. Furthermore, PTX3 sequesters fibroblast growth factors (i.e., FGF2, FGF8b) thus inhibiting their angiogenic and mitogenic activities (adapted from Bottazzi *et al* [7]).

PTX3 opsonises *Neisseria meningitides* and decreases the bacterial load in an infant rat model of meningococcal meningitis [302]. Interestingly, in this model, PTX3 amplifies the antibody response to vaccination, thus acting as a “natural adjuvant” of adaptive immunity

[302]. PTX3 binds to the outer membrane protein A (KpOmpA) of *Klebsiella pneumoniae* and amplifies the complement-dependent response to this bacterium via functional cooperation with cell-associated PRMs, including the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), the scavenger receptor expressed by endothelial cell-I (SREC-I), and TLR2 [303].

Evidence is growing to suggest that PTX3 can mediate antiviral activities both *in vitro* and *in vivo* against human and murine cytomegalovirus (HCMV and MCMV, respectively) and influenza virus type A (H3N2 strain) [304]. PTX3 binds both HCMV and MCMV and reduces viral entry and infectivity in DCs *in vitro* [149]. Consistently, PTX3-deficient mice are more susceptible to MCMV infection than their wild-type littermates, and PTX3 protects susceptible animals from MCMV primary infection and reactivation *in vivo* as well as *Aspergillus* superinfection [149]. Also, human and murine PTX3 bind influenza virus H3N2, where this interaction involves the viral hemagglutinin glycoprotein and the sialic acid residues present on the glycosidic moiety of the protein [304]. PTX3 inhibits the virus-induced hemagglutination and viral neuraminidase activity, and neutralizes the virus infectivity [304]. As opposed to these antiviral activities, recent studies indicate that PTX3 can promote rather than inhibit virus entry and replication in target cells. This is the case of arthritogenic alphaviruses (i.e., chikungunya and Ross River viruses), which highlights a potential contribution of this long pentraxin to the pathogenesis of alphaviral diseases [305].

Elevated circulating levels of PTX3 have been found in several systemic infections [306]. Additional studies pointed to PTX3 as a prognostic/diagnostic marker in a number of infectious diseases, including Dengue virus infections, pulmonary tuberculosis (TB), leptospirosis, meningococcal diseases, pulmonary aspergillosis, and urinary tract infections caused by UPEC [7]. Most interestingly, PTX3 genetic variants have been found to be associated with several infections in humans, including pulmonary tuberculosis, cystic fibrosis-related *Pseudomonas aeruginosa* infections, UPEC burdens, and IA in hematopoietic stem cells transplanted patients [307,301,300,308]. Overall, these reports support prophylactic, therapeutic, and diagnostic applications of PTX3 in infectious diseases [309].

1.6.3 CROSSTALK WITH THE COMPLEMENT SYSTEM

PTX3 is well established to interact with several components of three complement pathways, and functionally cooperate with them in *self/non-self* discrimination, tissue remodeling and immune homeostasis [310,311]. Prompted by the observation that the short pentraxins CRP and SAP both bind C1q [283], this recognition unit of the CP was the first complement protein to be identified as a ligand of PTX3. However, unlike the classical pentraxins, PTX3 interacts with C1q in a calcium-independent manner. Furthermore, the engagement of C1q has opposing functional outcomes, depending on the way PTX3 is presented: immobilized PTX3 induces CP activation with increased deposition of C3 and C4, whereas fluid-phase PTX3 inhibits the CP, possibly via competitive blocking of activation sites on C1q [207]. Both PTX3 and short pentraxins recognize late apoptotic cells, and competition experiments suggest that the three pentraxins may share common yet unknown ligands on their surface [312]. In particular, PTX3 shapes the C1q-dependent CP activation on apoptotic cells and their engulfment by professional phagocytes, with potential implications in autoimmunity and autoinflammation [313].

As previously discussed above (see section 1.3.2.2), PTX3 interacts both with ficolin-1 (M-ficolin) and ficolin-2 (L-ficolin) in a calcium dependent fashion, through their fibrinogen binding domain [86]. Ficolin-2 (but not ficolin-1) binds *A. fumigatus*, and PTX3 strengthens this interaction thus amplifying complement activation on this fungal pathogen. Recognition of ficolin-1 by PTX3 is mediated by the sialic acid moieties that are present on the long pentraxin oligosaccharides, and has been shown to trigger activation of the LP *in vitro* [314]. PTX3 also interacts with the collagen-like domain of MBL in a calcium dependent manner. Formation of this complex allows recruitment of C1q and enhances C4 and C3 deposition on and phagocytosis of *Candida albicans* [191].

PTX3 is able to interact with soluble regulators of both AP and CP/LP, such as FH and C4BP, respectively [4,315]. It has been reported that PTX3 binds to two specific sites on the FH protein: one is located in the complement control protein modules (CCPs) 19-20, that interact with the N-terminus of PTX3; the other is in the CCP 7 that binds the glycosylated C-terminus of the long pentraxin. In a complex with PTX3, FH is still able to exert its inhibitory activities [315]. In this regard, it has been described that PTX3 favours the recruitment of FH onto apoptotic cells and, in this way, the deposition of iC3b on their surface [315]. This is believed to prevent excessive complement activation and promote the phagocytic removal of apoptotic bodies [315]. Similarly to FH, PTX3 binds C4BP,

with no alteration of the cofactor activity of this fluid phase inhibitor of CP/LP [4]. In this regard, it has been observed that PTX3 recruits C4BP onto ECM and apoptotic cells, thus favouring the proteolytic cleavage of C4b and inhibiting formation of the CP/LP C3 convertase [237].

1.6.4 PTX3 AND *A. FUMIGATUS*

A. fumigatus is recognized by the innate immune system via both soluble and cell-associated PRMs (see “Innate immunity and *A. fumigatus*”) [150]. Amongst soluble PRMs, a major role has been attributed to PTX3 [2]. In this regard, *ptx3*-null mice have been shown to be highly susceptible to IA, due to defective recognition of conidia by PMNs, AMs, and DCs, and biased Th2 responses [141]. As described above, PTX3 is stored in the specific granules of neutrophils (see section 1.6.1), and neutrophils are essential for host resistance to *A. fumigatus* infections (section 1.3.1). In line with these observations, adoptive transfer of neutrophils from PTX3-deficient mice to PTX3-competent littermates previously infected with *A. fumigatus* results in increased fungal burden in the lung [98]. Perhaps more importantly, administration of the exogenous protein has therapeutic efficacy in several models of IA in immunocompromised hosts [316-318]. Also, exogenous PTX3 retains its protective activity in the combination therapy, where it enhances or even synergizes with clinically established antifungal drugs, like voriconazole [317]. Furthermore, polymorphisms in the *ptx3* gene are associated with reduced systemic levels of the protein and increased risk of IA in human stem cell transplant recipients [161], and invasive mold infections after solid organ transplant [319] and in acute leukemia patients on intensive chemotherapy [162]. Taken together, these observations point to a key role of PTX3 in the immune response to *A. fumigatus*, and strongly encourage the translation of this long pentraxin to the clinic, for prophylaxis and/or treatment of IA, either alone or in combination with antifungal drugs.

Recognition of *A. fumigatus* by PTX3 is restricted to resting, swollen and germinating conidia, with little or no interaction of the protein with fungal hyphae [141]. Based on competition experiments, galactomannans have been proposed as ligands of PTX3 on the conidial wall [141]. However, these polysaccharides are abundant in the hyphal wall too, and no direct interaction of PTX3 with purified galactomannans has been reported so far, which suggests that other cell wall components are recognized by this long pentraxin.

Also, while PTX3 binds conidia via its N-terminal region, it has been described that recombinant forms of this domain fail to promote phagocytosis of fungal conidia, indicating that the C-terminal pentraxin domain is somehow involved in this function [2].

Current literature indicates that PTX3 has opsonic activity towards *A. fumigatus*, and enhances recognition, phagocytosis and killing of fungal conidia by immune cells, mainly PMNs, via complement and Fc receptors pathways [2]. Activation of the complement system on the various morphotypes of *A. fumigatus* involves all three pathways, as previously discussed (see section 1.4), and PTX3 interacts with recognition and regulatory molecules of both CP/LP and AP (section 1.6.3). However, only the AP appears to be required for the pro-phagocytic and pro-killing activities of PTX3. Indeed, these are retained *in vivo* in C1q-deficient mice [141], and *in vitro* in human sera that had been depleted of C4, an essential component of the CP/LP C3 convertase [2]. PTX3 recognizes the major soluble inhibitors of the complement system (i.e., C4BP and, more importantly, FH), whose recruitment onto the fungal wall is regarded as a strategy of complement evasion [82,246]. However, if and how PTX3 affects this strategy is not known. Moreover, there is poor understanding of the molecular crosstalk between the complement system (i.e., AP), innate immune cells (i.e., PMNs) and this long pentraxin in the immune reaction to *A. fumigatus*.

2. AIMS OF THE PHD PROJECT

As described in section 1.6.4, PTX3 has been proposed as a functional ancestor of antibodies (“ante-antibody”): it has opsonic activity towards *A. fumigatus*, and enhances recognition, phagocytosis and killing of fungal conidia by immune cells, mainly PMNs, via complement and Fc receptors pathways [2].

This notwithstanding, the molecular mechanisms and structural determinants of PTX3 activity in the opsono-phagocytosis of *A. fumigatus* are at present unclear. This activity has been shown to require C3 and factor B (i.e., that form the C3 convertase of the AP), but not C1q (i.e., initiator of the CP) [2]. However, it is currently unclear whether this process is due to enhanced C3 deposition on conidia or to increased recognition of the PTX3-opsonised conidia by complement receptors (CRs) and FcRs, or both. *A. fumigatus* has developed several strategies to evade the immune response, such as attracting onto the conidial wall inhibitors of the complement system such as FH and C4BP [82,246]. PTX3 recognizes sites on these proteins (i.e., complement control protein (CCP) modules 7 and 19-20 in FH and α chain in C4BP) that mediate binding to *A. fumigatus* conidia [4,315]. Therefore, I hypothesized that this long pentraxin might affect the interaction of *A. fumigatus* with FH and C4BP, possibly rescuing full complement activation, and in this way enhancing phagocytosis and killing of *A. fumigatus* by neutrophils.

The human PTX3 protein is made of an N-terminal region and a C-terminal pentraxin domain, homologous to the short pentraxins CRP and SAP [7]. In addition to the multidomain organization, PTX3 has a complex quaternary structure with 8 disulphide-linked protomers folding into an elongated and asymmetric molecule [298]. The modularity and oligomeric nature of the PTX3 protein are likely essential for its functions in IA. Thus, I further hypothesized that PTX3 can establish multiple interactions with conidia, complement and effector cells, thus acting as a molecular bridge in bringing together target microbes and immune components.

In this PhD project, I challenged these hypotheses by implementing a multi-disciplinary approach that integrates techniques and methodologies of immunology, cell biology, protein biochemistry and the use of animal models, as described in the following chapters. Therefore, primary aim of the study was to characterize the molecular crosstalk between the complement system and the long pentraxin PTX3 in the immune response to

Aspergillus fumigatus infections. This was sought for by pursuing the following specific aims:

- to identify the complement components that are required for the opsono-phagocytic activity of PTX3;
- to characterize how PTX3 modulates complement activation on *A. fumigatus* conidia;
- to define the molecular mechanism of the PTX3/complement crosstalk;
- to characterize and better define the cell-associated receptors involved in the PTX3 activity;
- to elucidate the structure/function relationship of the PTX3 protein that support its pro-phagocytic activity towards *A. fumigatus*.

3. MATERIALS AND METHODS

3.1 PROTEINS AND ANTIBODIES

Recombinant human PTX3 was purified from a CHO 3.5 cell line stably and constitutively expressing the protein as described [320]. PTX3-2S and PTX3-5S mutants, made in the context of the full length protein and containing serine in the place of cysteine at positions 317/318 and 47/49/103/317/318, respectively, and the C-terminal domain of PTX3 (C_PTX3; residues 178–381 of the preprotein) were expressed in and purified from CHO cells [298]. The N-terminal domain of PTX3 (N_PTX3; residues 18–170) and its N_PTX3-3S mutant, bearing Cys/Ser substitutions at positions 47/49/103, were made in HEK-293F cells [321]. Purified complement proteins from human plasma were purchased from Complement Technology (Tyler, USA). Purified CRP from human plasma was from Merck Millipore (Burlington, USA). Normal human serum (NHS) and human sera depleted of selected complement proteins (DHS) were from Complement Technology. Goat α -human factor B polyclonal antibody (pAb) was purchased from R&D System (Minneapolis, USA). Rabbit α -human PTX3 polyclonal antibody (pAb), rat α -N_PTX3 and α -C_PTX3 monoclonal antibodies (mAbs) were made in-house as described [320]. Aliquots of rabbit α -human PTX3 pAb were biotinylated with NHS-LC-Biotin (Thermo Scientific, Waltham, USA), according to the manufacturer's instructions. Mouse α -human CRP mAb, goat α -human C3 and α -human FH pAbs were from R&D System. Goat α -human C4 pAb was from Complement Technology, mouse α -human C4c and α -human C4d mAbs were obtained by AbD Serotec (Kidlington, UK). Sheep α -human C4BP pAb was supplied by Abcam (Cambridge, UK). HRP-conjugated donkey α -rabbit IgG and α -mouse IgG pAbs were from GE Healthcare (Little Chalfont, UK); HRP-conjugated donkey α -goat IgG and α -sheep IgG pAbs were from R&D System. Mouse α -human CR1 (CD35) blocking mAb was from OriGene Technologies (Rockville, USA). This IgG₁ antibody (clone J3D3) has been reported to inhibit phagocytosis of *F. tularensis* by human neutrophils *in vitro*, where CR1 is strictly required for this process [101]. Rat α -Mouse CR3 (CD11b) blocking mAb (that recognizes human CR3) was obtained from BD Biosciences (Franklin Lakes, USA). This IgG_{2b} antibody (clone M1/70) has been shown to inhibit the CR3-mediated rosetting of EC3bi (erythrocytes bearing homogeneous human iC3b) by human macrophages and PMNs [322]. Mouse α -human CR4 (CD11c) blocking mAb was purchased from Novus Biologicals (Littleton, USA). This IgG₁ antibody (clone

3.9) has been described to inhibit the CR4-mediated phagocytosis and killing of *S. pyogenes* by human neutrophils [323]. Mouse α -human CD16 and CD32 blocking mAbs were from Ancell (Stillwater, USA). The α -human CD16 IgG₁ (clone 3G8) and α -human CD32 IgG₁ (clone 7.3) have been reported to inhibit the binding of soluble immune complexes to monocyte-derived dendritic cells [324]. Isotype control mAbs were from BD Biosciences and Proteintech (Rosemont, USA). Peridinin chlorophyll protein complex (PerCP) anti-mouse CD45, phycoerythrin (PE) anti-mouse Ly6G and brilliant violet 421 (BV421) mAbs were from BD Biosciences.

3.2 PROCEDURES INVOLVING ANIMALS

C57BL/6 mice were from Charles River Laboratories (Calco, Italy). PTX3-deficient mice were generated on a C57BL/6 background as described [141]. C57BL/6 C3-deficient, C57BL/6NJ factor B-deficient and C57BL/6NJ wild type mice were from The Jackson Laboratory (Bar Harbor, USA). Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council 1996). All efforts were made to minimize the number of animals used and their suffering.

3.3 PREPARATION OF FUNGAL CONIDIA

The *A. fumigatus* (AF) strain used in this study is a clinical isolate from a fatal case of pulmonary aspergillosis [134,2]. Conidia were harvested in PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137.9 mM NaCl, 8.1 mM Na₂HPO₄, pH 7.0) containing 0.05% (v/v) Tween 80 from 7-10 day old cultures grown on Petri dishes containing 2% (w/v) malt agar extract solid medium [134]. Harvested conidia were filtered on sterile gauze, extensively washed with PBS and counted on an inverted microscope. Following heat inactivation at 95°C for 2 hours, concentration was adjusted to 10⁹ conidia/ml, and heat-inactivated conidia were stored at 4°C until use. For selected experiments (binding, phagocytosis and killing) viable AF conidia were used immediately after harvest.

3.4 FLOW CYTOMETRY BINDING ASSAYS

AF conidia were incubated with the indicated concentrations of either PTX3, N_PTX3 or C_PTX3 in PBS⁺ (0.9 mM CaCl₂, 0.49 mM MgCl₂ in PBS) containing 0.1% (v/v) Tween 20 for 1h at room temperature (RT) on an rotating wheel. After extensive washing, bound proteins were revealed with biotinylated rabbit α -human PTX3 pAb (0.5 μ g/ml) followed by Alexa 647-streptavidin (BioLegend, San Diego, USA; 1:500) on a FACS Canto I flow cytometry system (BD Biosciences, East Rutherford, USA). Data were acquired and analysed using the FACS Diva software (BD Biosciences) and results were expressed as both event counts and mean fluorescence intensity (MFI).

3.5 MICROTITER PLATE BINDING ASSAYS

FH and C4BP binding to PTX3, N_PTX3 and C_PTX3 was assessed by ELISA using a method described in [297]. Briefly, 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were coated with the indicated amounts of PTX3 proteins, blocked with 2% (w/v) bovine serum albumin (BSA) in HBS (20 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, pH 7.0) containing 0.1% (v/v) Tween 20 (HBS-T), and incubated with FH and C4BP (both at 17 nM). Bound proteins were detected using the appropriate primary and HRP-conjugated secondary pAbs, followed by the 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic substrate (Sigma Aldrich, Saint Louis, USA). Plates were read on a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, USA) and results were expressed as absorbance at 450 nm (A_{450nm}) following subtraction of the background signal from empty wells (i.e., without PTX3 proteins).

Binding of proteins to AF conidia was assessed using 96-well HTS Multiscreen filter plates (Merck Millipore, Darmstadt, Germany), equipped with 1 μ m PVDF bottom membranes (that allow retention of conidia and are permeable to soluble molecules) and operated by a vacuum manifold (Figure 7). Plates were pre-wet with 200 μ l/well PBS⁺, and saturated with 200 μ l/well PBS⁺ containing 1% (w/v) non-fat dry milk at 37°C for 2h. Conidia (10⁶ per well) were then applied and incubated at RT for 1h with a range of protein concentrations in HBS-T. Plates were washed with 200 μ l/well HBS-T, and conidia-bound proteins were revealed with the appropriate primary and HRP-conjugated secondary mAbs and pAbs in HBS-T containing 0.5% (w/v) BSA. TMB (150 μ l/well) were then added and allowed to react for 10-15 min; 80 μ l/well reaction solution were transferred into Maxisorp plates and colour development was stopped by addition of 40

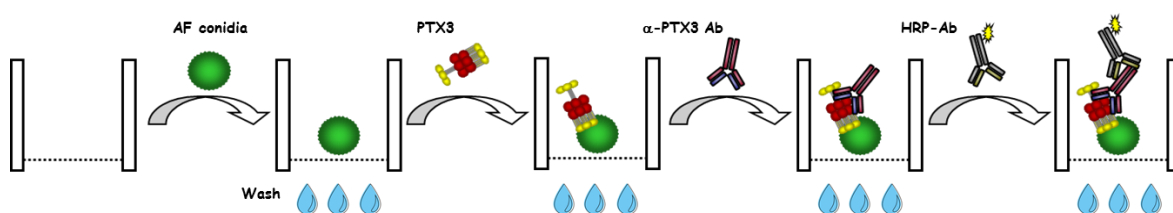


FIGURE 7. Schematic representation of the binding assays performed on microtitre filter plates. As an example, the major steps of a typical binding experiment aimed at assessing the interaction of PTX3 with AF conidia are shown.

μl/well 2N sulphuric acid. Plates were read on a VersaMax spectrophotometer and results were expressed as $A_{450\text{nm}}$. Background absorbance from empty wells (i.e., without conidia) was subtracted at each protein concentration. Sequential incubation binding assays were also performed, where conidia were pre-incubated with saturating concentrations of proteins (as inhibitors), prior to addition of selected ligands. In another set of experiments, conidia were co-incubated with a range of molar ratios of inhibitors over ligand. In all cases, colorimetric detection was performed as described above and results were expressed as either absorbance at 450 nm or percentage of the free ligand binding.

3.6 KINETIC ANALYSES

Kinetics of the interaction with AF of PTX3 (and its mutant constructs) and FH was studied using real-time label-free approaches. In this regard, Quartz Crystal Microbalance (QCM) and Surface Plasmon Resonance (SPR) are both powerful technologies that are receiving increasing attention in as much diverse fields as biomedicine, material science and food safety/quality assurance. The physical principles on which these technologies are built upon are briefly introduced in this chapter (see [325] for extensive reviewing of QCM and SPR, respectively, and [326] and [327] for comparative studies on both technologies).

QCM is in essence a mass loading sensor-based technology that allows weighing nanograms/sub-nanograms of material. The most critical component of a QCM system is the quartz crystal that is cut in the so-called AT orientation to form keyhole shaped disks, or chips. The chip surface is patterned with electrodes, which serve two purposes: 1) to drive the crystal into oscillation or resonance electrically and 2) provide a sensor surface on which molecular binding takes place. The most pronounced oscillation or resonance mode is the shear mode (displacement along the chip surface), and its frequency decreases

upon adsorption of molecules onto the sensor surface. The frequency change (Δf) is proportional to the mass change of adsorbed molecule/s per unit area (Δm), as analytically expressed in the Sauerbrey equation:

$$\Delta f = -\frac{1}{C_{QCM}} \Delta m$$

where C_{QCM} is the mass sensitivity and equals to $V\rho/2f^2$ (f is the resonance frequency, V and ρ are the shear module and density of quartz, respectively).

SPR is based on a different principle, however it also provides the mass of molecule/s adsorbed on a sensor chip, a glass slide covered with a thin layer of metal (e.g., Au). In a typical SPR setup, a light beam is directed onto the metal film through an optical prism and surface plasmons in the metal film are excited if the incident angle (resonance angle) is appropriate. The resonance angle is extremely sensitive to polarizability and density of the adsorbed molecule/s. For most biological molecules (e.g., proteins), polarizability is fairly constant, so shifts in the resonance angle are proportional to the mass of the adsorbed molecule/s, as expressed in the equation:

$$\Delta\theta = \frac{1}{C_{SPR}} \Delta m$$

where C_{SPR} is the mass sensitivity for SPR.

The key performance parameters of both technologies are listed, for comparative purposes, in Table 1. As shown in the table, SPR is clearly superior to QCM in terms of mass sensitivity, limit of detection, insensitivity to macromolecule-associated water, and throughput (i.e., flow cell number and volume). However, cell-based assays are problematic with SPR systems. In this regard, it is not possible to immobilize/capture intact cells on SPR chips, likely due to the small size of the flow cell. Also, although studies have been performed where cells have been flown over ligand-coated surfaces [328], this experimental setup does not fully reproduce the natural biological frame of a biomolecular interaction (e.g., binding of a soluble ligand to a cellular receptor). QCM, instead, is more amenable than SPR to cell-based assays, due to larger flow cell volumes and microfluidic channels. In addition, QCM chips can be coated with sterile polystyrene, which allows culturing live cells in standard laboratory incubators. Therefore, despite the limitations of QCM, the interaction with AF conidia of PTX3, PTX3-2S, PTX3-5S,

N_PTX3, N_PTX3-3S, C_PTX3 and FH was investigated using QCM Attana Cell 200 system (Attana AB, Sweden).

TABLE 1. Major performance parameters of QCM and SPR. Values are from [326] and technical information available from GE Healthcare Life Sciences (www.gelifesciences.com), Accela (www.accela.eu/attana/cell-200) and Biolin Scientific (<https://www.biolinscientific.com/qsense>).

Parameter	QCM	SPR
Mass sensitivity ^a	~20 ng/cm ²	~1 ng/cm ²
Limit of detection ^b	~2 ng/cm ²	~0.1 ng/cm ²
Flow cell volume ^c	1460 nL	40-60 nL
Flow cell number ^c	up to 8	up to 36
Chip surface functionalization ^d	excellent	excellent
Water displacement effects ^e	sensitive	insensitive
Viscoelastic effects ^f	sensitive	insensitive
Cell-based assays	Suitable	not or poorly suitable

^a Mass sensitivity is defined as the mass of detected molecule/s per unit area of a sensor surface for a given change of the sensor signal (i.e., frequency in QCM, and angular shift in SPR). The reported values refer to a frequency change of 1 Hz (for QCM) and an resonance angle change of 1 mdeg (for SPR).

^b Limit of detection (LOD) is defined as the smallest amount of molecule/s adsorbed onto a sensor surface that can be detected. LOD is determined by mass sensitivity, response time and sensor signal resolution. The reported values refer to a frequency resolution of 0.1 Hz (for QCM) and an angular resolution of 0.1 mdeg (for SPR) for a response time of 1 s.

^c SPR systems typically comprise microfluidic flow channels that are built on site by pressing a patterned gasket onto a sensor surface. This allows generating a multiplicity of flow cells with low internal volume. Similar fluid handling strategies cannot be implemented for QCM, due to the inherent physical properties of this technology (electromechanical rather than optical), therefore QCM systems have fewer flow cells with larger internal volume.

^d Sensor chip surfaces need to be pre-functionalized with appropriate layers of ligands/probes or organic matrices to reduce non-specific adsorption. QCM and SPR systems share common surface chemistries for chip functionalization, as they both use gold-coated chips.

^e SPR is not sensitive to macromolecule-associated water (hydration), and the resonance signal can be directly converted to the molecular mass of the adsorbed macromolecule/s. Contrary to that, QCM measures the molecular mass of the adsorbed macromolecule/s together with the associated water. The complexity in mass measurement however does not impair QCM's suitability for kinetic evaluation of binding reactions.

^f Dissipation over time (D) of the QCM signal (as measured in QCM-D systems) provides valuable insights into the surface structure, viscoelastic properties, and conformational changes in the adsorbed macromolecule/s upon binding. This cannot be attained with SPR systems.

Heat-inactivated AF conidia (10^8) were seeded and adsorbed onto cell compatible sensor chips in 10 mM acetic acid, pH 4.0 for 3h at RT, and free sites were blocked with 100 μ g/ml BSA in the same buffer for 3h at RT. Sensor chips were extensively washed with PBS to remove unbound conidia prior to docking into the Attana Cell 200 system. A control chip was made that contained BSA only. Following baseline stabilization, PTX3 proteins and FH were injected at a range of concentrations in PBS (flow rate 25 μ l/min, injection time 84 s, dissociation time 1500 s), and QCM frequency (in Hertz) was recorded as a function of time (s). Global fitting was performed on reference and buffer-subtracted sensorgrams using the Attache Evaluation Software (Attana AB), and values were measured for the kinetic rate constants k_{on} and k_{off} ; the equilibrium dissociation constant (K_D) was calculated from the k_{off}/k_{on} ratio.

In separate QCM experiments, the effect of PTX3 pre-opsonisation on FH binding to AF conidia was investigated. Briefly, FH was injected onto AF-coated chips; following regeneration of the chip surface, PTX3 was applied prior to an additional injection of FH. Frequency shifts (in Hertz) were recorded under steady state conditions, and the percentage of residual FH binding (after injection of PTX3) was calculated.

3.7 SIZE EXCLUSION CHROMATOGRAPHY

Solutions of PTX3 (at 60 μ g/ml), C3b (at 200 μ g/ml) and FH (at 200 μ g/ml) in PBS⁺ containing either the individual proteins or different binary and ternary combinations of them were incubated for 30 minutes at 37°C prior to loading onto two Superose 6 HR 10/300 SEC columns mounted in series on an AKTA Purifier chromatography system (GE Healthcare Life Sciences, Pittsburgh, USA), equilibrated and eluted at 0.5 ml/min with PBS⁺. Protein separation was monitored by UV absorbance at 280 and 220 nm and 1.5 ml fractions were collected and analysed using NuPAGE Novex Bis-Tris 10% gels (Thermo Fisher Scientific, Waltham, USA) under denaturing and reducing conditions (i.e., in the presence of dithiothreitol). Following electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for subsequent immunodetection with a mixture of α -PTX3, α -FH and α -C3 pAbs, followed by a mixture of anti-rabbit and anti-goat IgG horseradish peroxidase (HRP)-linked whole donkey antibodies (GE Healthcare), for simultaneous detection of the three proteins on the same blots. Membranes were developed with Immobilon western HRP substrate (Merck Millipore) and chemiluminescence recorded on a Chemidoc MP system (Bio-Rad, Hercules, USA).

To rule out potential aggregation artifacts in the QCM experiments (see sections 3.6 and 4.5), further analyses were carried out on N_PTX3 preparations. Briefly, 100 μ l of 1 mg/ml solutions of N_PTX3 in PBS⁺ were chromatographed on a Superdex 200 GL 10/300 SEC column, equilibrated and eluted with PBS⁺ at 0.5 ml/min. Protein separation was monitored by UV absorbance at 280 nm. In addition, aliquots of unfractionated N_PTX3 solutions were run under denaturing conditions on 4-12% (w/v) Bis-Tris gels (Thermo Fisher Scientific), in the absence (- DTT) and presence (+DTT) of dithiothreitol. Following separation, protein bands were stained with Bio-Safe™ Coomassie (Bio-Rad), and gel images recorded on a Chemidoc MP system (Bio-Rad).

3.8 ASSAY FOR THE FH COFACTOR ACTIVITY (HYDROLYSIS OF C3B TO IC3B)

The effect of PTX3, its domains and corresponding mutant constructs on the co-factor activity of conidia-bound FH and C4BP was evaluated by an adaptation of previously described methods [315,4]. Briefly, AF conidia (3.5×10^8 /ml in a final volume of 300 μ l) were pre-incubated with either PTX3, PTX3-5S, PTX3-2S, N_PTX3, N_PTX-3S or C_PTX3 (all at a molar equivalent concentration of 60 nM), prior to addition of saturating concentrations of either FH (133 nM) or C4BP (35 nM). Unbound proteins were removed with HBS-T and mixtures of either C3b and FI or C4b and FI (all at 75 nM) were applied, and proteolysis was terminated with 50 μ l 100 mM EDTA (Figure 8). All incubations were performed in HBS-T at 37°C for 1h on a thermal mixer (at 900 rpm). Reaction mixtures were centrifuged at 13,000 g for 5 min and conidia-free supernatants were loaded onto 4-12% Bis-Tris NuPAGE gradient gels (Thermo Fisher Scientific), following thermal denaturation and reduction at 70°C for 10 min. After electrophoretic separation, proteins were electroblotted onto 0.45 μ m PVDF membranes and revealed with either goat α -human C3 pAb (1:5,000) or goat α -human C4 pAb (1:25,000), followed by the appropriate HRP-conjugated secondary pAbs. Blots were developed using the Immobilon Western kit (Merck Millipore) and chemiluminescence was recorded on a ChemiDoc MP system (Bio-Rad). Acquired images were analyzed with the ImageLab software (Bio-Rad), and local density background was subtracted from band volumes. Experiments performed in the absence of FI were taken as negative controls.

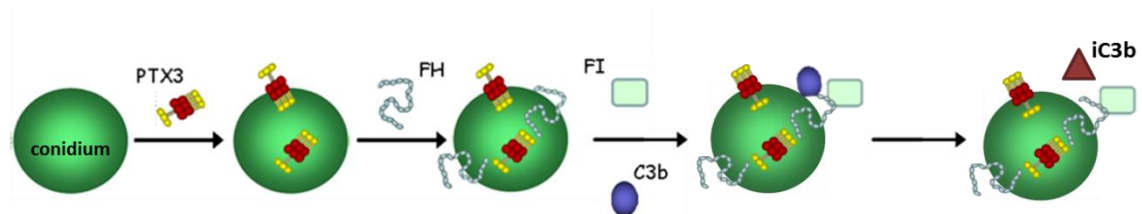


FIGURE 8. Schematic of the assay for C3b inactivation (i.e., cleavage to iC3b) on AF conidia by FH and FI, in the presence of PTX3. Similar experiments were performed on C4BP (in the place of FH) and C4b (in the place of C3b).

In some experiments, at termination of proteolysis, conidia were transferred onto 96-well HTS Multiscreen filter plates and bound C4c and C4d fragments were revealed with the corresponding mouse mAbs, as described above. Results were expressed as C4c/C4d absorbance ratios and normalized based on values obtained in the absence of the PTX3 proteins.

3.9 ASSAY FOR THE FH INHIBITION/DECAY-ACCELERATING ACTIVITY (INHIBITION/DISSOCIATION OF THE C3 CONVERTASE)

To assess the effect of PTX3 proteins on the C3 convertase inhibition/decay-accelerating activities of conidia-bound FH and C4BP, AF conidia were sequentially incubated on microtitre filter plates with PTX3 (60 nM) and mixtures of either C3b, FB and FH (111 nM, 38 nM and 55 nM, respectively) or C4b, C2 and C4BP (104 nM, 43 nM and 15 nM, respectively). Bound C3b, FH, C4b and C4BP were revealed with goat α -human C3 (1:5,000), goat α -human FH (1:5,000), goat α -human C4 (1:25,000) and sheep α -human C4BP (1:3,000) pAbs, respectively, followed by the appropriate HRP-conjugated secondary pAbs, as described above.

3.10 C3 PROTEOLYSIS AND DEPOSITION ON AF CONIDIA

To evaluate the effect of PTX3 on deposition of C3 (and its fragments) on AF, heat inactivated conidia were pre-incubated with the PTX3 proteins in HBS-T. Conidia (1×10^9 /ml) were washed to remove unbound proteins and further incubated with solutions of AP proteins at 5% (v/v) of their serum concentration (25 μ g/ml FH; 1.7 μ g/ml FI; 10 μ g/ml FB; 0.25 μ g/ml FP; 0.07 μ g/ml FD; 60 μ g/ml C3) in 100 μ l GVB^{+/+} (Gelatine

Veronal Buffer; 5 mM Veronal, 145 mM NaCl, 0.5 mM MgCl₂, 0.15 mM CaCl₂, pH 7.30, containing 0.1% (v/v) gelatin) on a thermal mixer at 900 rpm and 37°C. AP activation was blocked at different time points with an equal volume of 100 mM EDTA, pH 7.00. Conidia were washed with HBS-T-EDTA (HBS-T containing 10 mM EDTA), and bound C3 fragments were eluted with 50 µl 1 M NH₂OH, 0.2 M NaHCO₃, pH 10.0 for 1h at 37°C, and run on 4-12% Bis-Tris NuPAGE gels (Thermo Fisher Scientific). Proteins were transferred onto PVDF membranes and probed with a goat α-human C3 pAb. Blots were developed using the Immobilon Western kit (Merck Millipore) and chemiluminescence was recorded on a ChemiDoc MP system (Bio-Rad) (see Figure 9). Densitometric analyses were performed on the acquired images using the ImageLab software (Bio-Rad), and results were expressed as band volumes normalized based on local background. In parallel experiments, either FH- or FI-depleted human serum (FHDHS and FIDHS; 5% (v/v) in GVB^{+/+}) were used in the place of reconstituted AP. A similar approach was exploited to assess deposition of C4 and its fragments onto AF conidia, where NHS (5% (v/v) in GVB^{+/+}) was applied as a source of C4, and a goat α-human C4 pAb was used to reveal C4 fragments.

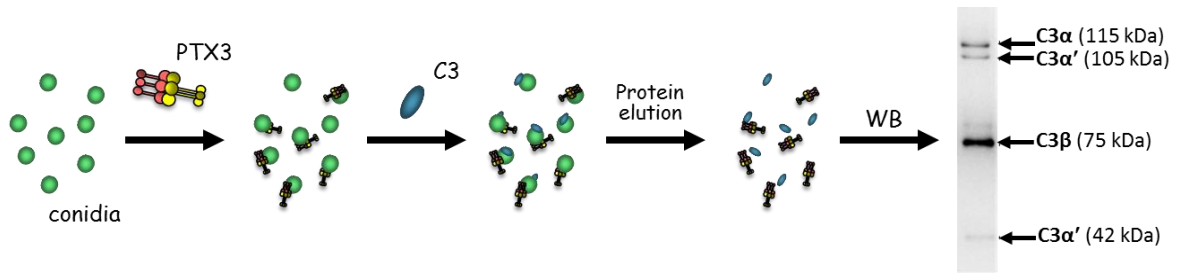


FIGURE 9. Experimental design for the assessment of C3 proteolysis and deposition on AF conidia and the effect of the PTX3 proteins on these processes. In alternative experiments, C4 deposition was monitored, using NHS in the place of reconstituted AP as a source of complement.

3.11 HUMAN PMNS ISOLATION AND PHAGOCYTOSIS *IN VITRO*

Human PMNs were freshly isolated from the peripheral blood of healthy volunteers, who signed an informed consent approved by the Institutional Review Board of Humanitas Research Hospital, as previously described [2]. Prior to phagocytosis, FITC-labelled AF conidia were pre-incubated with the PTX3 proteins in PBS⁺ containing 0.1% (v/v) Tween 20 for 1h at 37°C. Unbound proteins were removed with PBS⁺, and conidia (8x10⁶/ml) were further incubated with purified PMNs (1x10⁶ cells/ml; [conidia]:[PMNs] =8:1) for 30

min at 37°C, in the presence of 5% (v/v) either NHS or DHS in 0.2 mL RPMI 1640 (Thermo Fisher Scientific) or in the presence of solutions of AP proteins at 5% (v/v) of their serum concentration or selected components of AP (20 µg/ml C3b, 25 µg/ml FH, 1.7 µg/ml FI). Samples were placed on ice to block phagocytosis, and an equal volume of 250 µg/ml trypan blue (TB) in 0.1 M sodium citrate, pH 4.0 was added immediately before flow cytometry analysis. In selected experiments, PMNs were pre-incubated for 1 hour on ice in the presence of C3b, iC3b, blocking antibodies to CD35, CD11b, CD11c, CD16, CD32, or isotype controls (all at 10 µg/10⁶ cells).

In vitro phagocytosis experiments were purposely performed using heat-inactivated AF conidia, due to microbiology safety restrictions in place in our Department, and previous evidence indicating that heat-inactivation of AF conidia does not affect the pro-phagocytic activity of PTX3 [2]. However, for validation purposes, additional experiments were carried out using viable AF conidia (see the “Results” chapter), which were confined to the microbiology laboratory.

3.12 ANALYSIS OF PHAGOCYTOSIS BY FLOW CYTOMETRY

Phagocytosis was assessed by flow cytometry on a FACS Canto II system (BD Biosciences). In the presence of TB (that does not permeate live PMNs), FITC-labelled conidia lose their green fluorescence (by quenching) and acquire red fluorescence (by Fluorescence Resonance Energy Transfer, FRET), which allows the discrimination of phagocytosed conidia (that do not bind TB and retain green fluorescence) from those adhered to the cell membrane (which bind TB and become red fluorescent) [6]. Therefore, green fluorescence from FITC-labelled conidia and red fluorescence from TB bound to FITC-labelled conidia were recorded using 530±30 nm and 650±13 nm bandpass filters, respectively. PMNs were gated based on forward and side scatter. Data were acquired and analysed using the FACS Diva software (BD Biosciences) and results expressed as percentage of phagocytosis (PP; percentage of FITC-positive PMNs, excluding cells that had red fluorescence only). Data were expressed as mean ± SEM, where statistical analyses (Student’s paired t test) were performed on PP values obtained from 3-6 different donors for experimental condition and from at least 3 independent experiments.

3.13 KILLING ASSAY

The effect of PTX3 on the PMN-mediated killing of AF conidia was assessed as follows. Briefly, viable AF conidia were pre-incubated with PTX3, then further incubated with either NHS, FH-depleted human sera (FHDHS) or FH-supplemented FHDHS in the absence and presence of PMNs freshly isolated from the peripheral blood of healthy volunteers (1×10^6 cells/ml; [conidia]:[PMNs] = 8:1). At termination of incubation, PMNs (where present) were lysed with 50 mM Tris-HCl, 2 mM EGTA containing 1% (v/v) Triton X-100, and conidia viability was measured using the Alamar Blue (AB) assay (Thermo Fisher Scientific). More precisely, AB was added to the reaction vessels following lysis of PMNs, and allowed to react for 17 hours at 37°C. Vessels containing AF conidia only (which are not affected by the lysis treatment) were taken as a reference. Due to the oxidative metabolism of the growing spores, resazurin (a fluorogenic substrate that is a component of the AB kit) is reduced to resorufin, a red fluorescence light emitter. Background fluorescence in the absence of conidia was subtracted, and results were expressed as mean \pm SEM of the emitted fluorescence intensity (excitation 530-560 nm; emission 590 nm).

3.14 *IN VITRO* PHAGOCYTOSIS IN MURINE WHOLE BLOOD

In a separate set of experiments, phagocytosis of AF conidia by murine PMNs was assessed using whole blood from PTX3-, C3- and factor B-deficient mice, and their wild type littermates, which was collected in BD Vacutainer Heparin Tubes (BD) containing 17 IU heparin per ml of blood. In this regard, it is worth noting here that, although heparin selectively interacts with the coagulation system, it also has inhibitory effects on the classical [329] and alternative [330] pathways of complement. However, these are overcome by raising the temperature to 37°C [331]. Therefore, heparin-treated blood samples were initially kept at 4°C (to allow transfer from the animal facility to the laboratory, typically within 10 to 15 mins from collection), and the phagocytosis experiments were performed at 37°C, adapting a previously published procedure [2]. Briefly, 5×10^6 heat inactivated FITC-labelled conidia that had been pre-opsonised with recombinant human PTX3 (20 µg/mL) were incubated with a mixture of purified human C3b and FH (20 µg/ml and 8.5 µg/ml, respectively), prior to addition to 100 µl of whole blood and 30 min incubation at 37°C. Samples were placed on ice to block phagocytosis, and red cells were lysed with 2 ml of ice-cold ammonium chloride lysis solution at pH 8.0.

Samples were fixed with 1% paraformaldehyde (PFA) and analysed by flow cytometry using a combination of PerCP anti-mouse CD45, PE anti-mouse Ly6G and BV421 anti-mouse CD11b mAbs as previously described [2].

3.15 OPSONO-PHAGOCYTOSIS *IN VIVO*

C57BL/6 mice were anesthetized with ketamine (100 mg/Kg) and xylazine (20 mg/Kg) and injected intra-nasally (i.n.) with 5×10^7 heat inactivated FITC-labelled conidia that had been pre-incubated with either PTX3, PTX3-2S or sterile saline. Animals were sacrificed 4h after infection and the bronchoalveolar lavage (BAL) fluid was taken (see Figure 10). Conidia phagocytosis by BAL PMNs was analysed by flow cytometry using a combination of peridinin chlorophyll protein complex (PerCP) anti-mouse CD45 and phycoerythrin (PE) anti-mouse Ly6G mAbs as previously described [134,2].

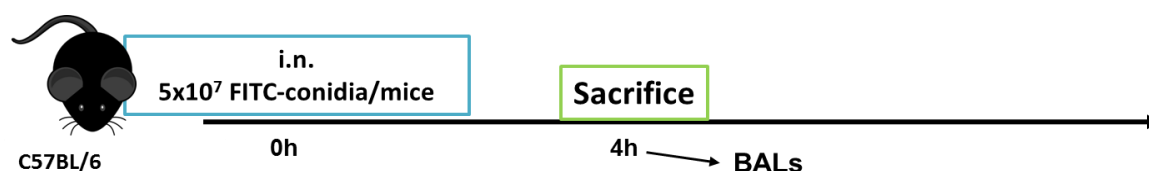


FIGURE 10. Outline of the opsono-phagocytosis experiments *in vivo*.

3.16 STATISTICAL ANALYSIS

Normality of the values recorded for the variables of interest (i.e., PP, absolute and relative amounts of C3 and C4 species, MFI, absorbance) was assessed by formal statistical testing (skewness and Shapiro-Wilk normality tests) and, whenever appropriate depending on sample size, graphical evaluation (kernel density, standardized normal probability, and quantile-quantile plots). The distribution of normal continuous outcome variables (e.g., PP) across two experimental groups (e.g., presence and absence of PTX3, taken as dichotomous exposure variable) was evaluated by either unpaired or paired Student's t test, depending on observations being unmatched or matched, respectively. In this regard, it is worth noting here that paired statistics was extensively used in data analysis because my data mostly consisted of pairs of outcome observations (e.g., PP values in a group of PMNs from different donors that were treated or not with PTX3), therefore it was important to take data pairing into account to assess how much on average the treatment (i.e., with PTX3) affected the outcome (i.e., PP). The effect of variables with three or more levels of

exposure on normal continuous outcome variables (e.g., the combined effect of PTX3 and C3b+FH treatments on AF conidia phagocytosis, as described in Figure 25) was assessed by analysis of variance, using one-way ANOVA with Dunnett's multiple comparison test as post-hoc analysis. In some experiments, the effect of two variables on the outcome was investigated (e.g., the effect of time and treatment with PTX3 on PP in Figures 18 and 19). Data generated in these experiments had a paired balanced design (i.e., equal numbers of matched observations in each group) and were analysed using the paired Student's t test, which, in this case, is equivalent to the two-way analysis of variance (see Kirkwood and Sterne, Essential medical statistics, 2th edition, Blackwell Science, Malden, USA). In all cases, values of $p < 0.05$ (not corrected for multiple testing) were taken as significant. Statistical analysis was performed using the GraphPad Prism 5.04 package (www.graphpad.com, GraphPad Software, San Diego, US).

4. RESULTS

4.1 INVESTIGATING ROLE OF SERUM, AS A SOURCE OF COMPLEMENT, IN THE PTX3-MEDIATED PHAGOCYTOSIS OF *A. FUMIGATUS* CONIDIA BY HUMAN PMNs

A major aim of this study was to investigate the molecular mechanisms underlying the role of PTX3 in the phagocytosis of *A. fumigatus* (AF) conidia by PMNs (see sections 1.6.4 and 2”). To this end, I first set up an *in vitro* phagocytosis assay that allowed discrimination of truly phagocytosed conidia (green circles in Figure 11A) from those adherent to the cell membrane or partially engulfed in the phagocytic cup (red and yellow circles in Figure 11A, respectively). This assay was adapted from a previously reported flow cytometry method [6] to provide unambiguous and operator-independent measurements of the percentage of phagocytosis (PP) (see section 3.12 in “Materials and Methods”). As shown in Figure 11B, PMNs phagocytosed AF conidia at a relatively low rate in the absence of normal human serum (NHS; PP of 9.2 ± 6.8), where this is likely mediated by serum-independent phagocytic receptors (see section 1.3.1), and PP linearly increased with the applied concentration of NHS (16.7 ± 13.0 , 23.7 ± 11.7 , 32.5 ± 1.7 at 5, 10 and 20%, respectively), regardless of the presence of PTX3. This long pentraxin had no effect on AF phagocytosis in the absence of NHS, indicating that serum is required for its pro-phagocytic activity, as previously reported [2]. However, pre-incubation of conidia with a range of PTX3 concentrations (0.6, 3 and 15 nM, corresponding to 0.2, 1 and 5 $\mu\text{g/ml}$, respectively, based on molecular weight (MW) of the PTX3 octamer, i.e. $\sim 340,000$, [298]) resulted in a significant increase in the observed PP values at any applied concentration. In particular, when the lowest concentration of PTX3 (0.6 nM) was used PP values of 21.4 ± 20.2 , 30.3 ± 18.1 and 41.7 ± 10.3 (at 5, 10 and 20% NHS, respectively) were observed. This is consistent with previous reports [2], furthermore it indicates that concentrations as low as 0.6 nM (compared to the lowest active concentration of 33.75 nM reported in [2]) are sufficient for PTX3 to promote AF phagocytosis by PMNs *in vitro*. In addition, this effect was dose-dependent when 5% NHS was used and saturated by 3 nM PTX3 (PP of 25.2 ± 22.9). Given that in healthy subjects the plasma levels of PTX3 are around 2 ng/ml (0.006 nM) and increase rapidly up to 1-2 $\mu\text{g/ml}$ (3-6 nM) in severe inflammatory and infectious conditions [332], most of the protein is synthesized locally at

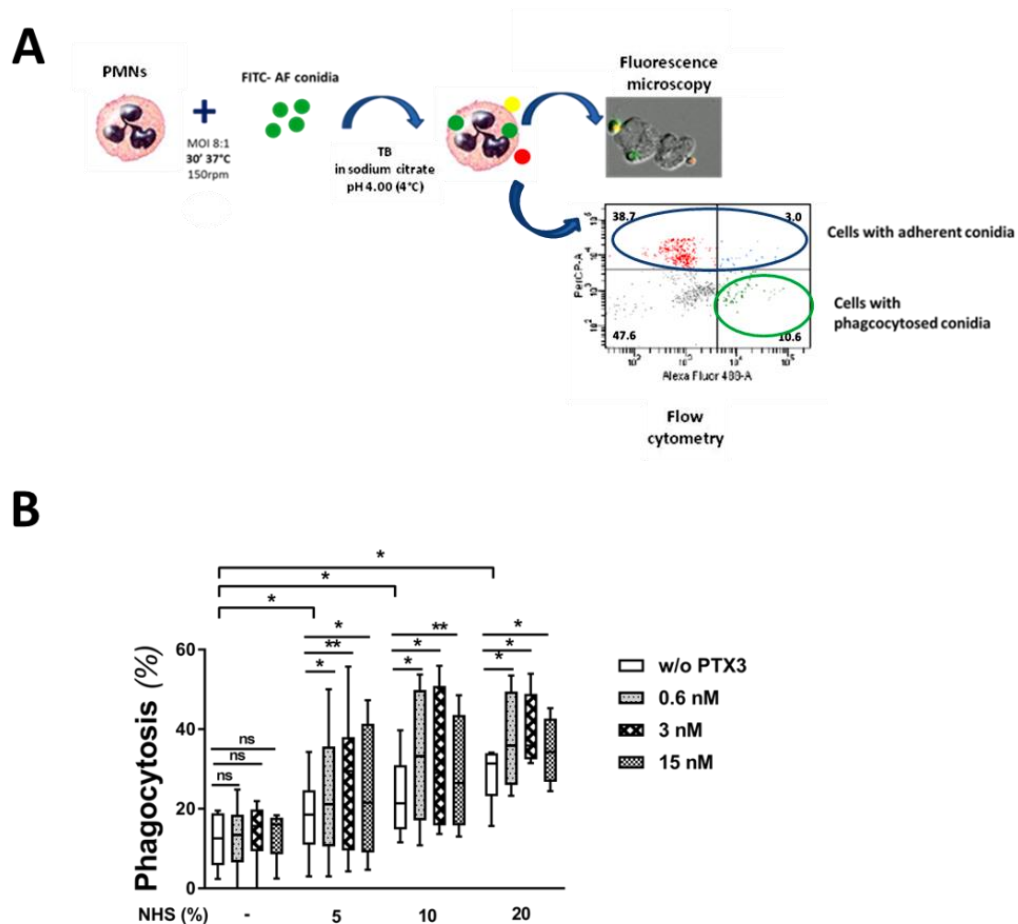


FIGURE 11. *In vitro* phagocytosis. **A)** Schematic diagram illustrating the experimental strategy used to assess phagocytosis of AF conidia by PMNs *in vitro*. Human PMNs freshly isolated from the peripheral blood of healthy volunteers were challenged in the presence or absence of NHS with heat-inactivated FITC-labelled AF conidia. Prior to flow cytometry analysis, a solution of trypan blue (TB, which does not permeate live cells) in sodium citrate at pH 4.00 was added. Under these conditions, FITC-labelled conidia lose their green fluorescence (by pH quenching) and acquire red fluorescence (by FRET), which allows discrimination of phagocytosed conidia (green circles, which do not bind TB and retain green fluorescence) from those that adhere to the cell membrane (red circles, which bind TB and become red fluorescent) or are halfway in the phagocytic process (yellow circles, which emit both green and red fluorescence) [6]. A fluorescence microscopy image and a FACS plot from a representative experiment are shown to illustrate the three different scenarios. In the case of FACS analyses, phagocytosis was assessed by flow cytometry as described in section 3.12 and results were expressed as percentage of phagocytosis (PP; percentage of FITC-positive PMNs [green dots in the lower right quadrant only, encompassed by the green oval], excluding cells that had red fluorescence [red and yellow circles in both upper quadrants, encompassed by the blue oval]). Numbers indicate the percentage of cells in each quadrant. **B)** Phagocytosis experiments were performed as described in **A**, where AF conidia that had been pre-incubated with a range of PTX3 concentrations (0.6, 3 and 15 nM) were used to challenge PMNs at a [conidia]:[PMNs] ratio (or multiplicity of infection, MOI) of 8:1, in the presence or absence of 5, 10 or 20% NHS. Results are expressed as PP values in the form of box and whisker plots from 4 independent experiments, each performed on a different donor (4 donors in total) in duplicate (** $P < 0.01$, * $P < 0.05$, Student's paired t test).

sites of inflammation/infection [98], and concentration of complement proteins in the human lung epithelium is likely lower than that in serum [333], I used 5% NHS and 3 nM PTX3 in subsequent phagocytosis experiments, unless otherwise stated.

4.2 COMPLEMENT PATHWAYS AND COMPONENTS THAT ARE REQUIRED FOR THE PRO-PHAGOCYtic ACTIVITY OF PTX3

A previous study by Moalli *et al.* [2] indicated that the pro-phagocytic activity of PTX3

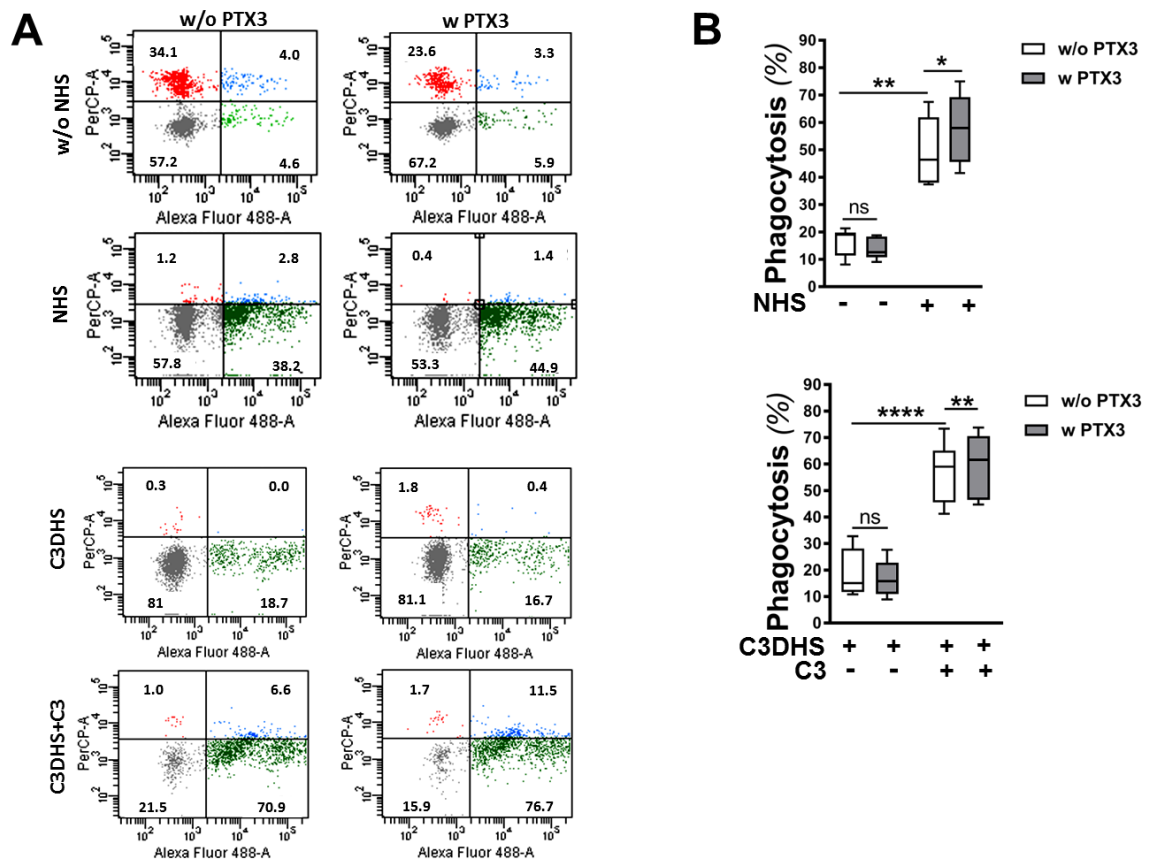


FIGURE 12. The pro-phagocytic activity of PTX3 is Complement-dependent. Phagocytosis was performed as described in Figure 11 in the presence or absence of 5% NHS and human sera depleted of C3 (C3DHS; that lack the opsonic activity of C3 and do not form the AP C3 convertase), and in the presence (w) or absence (w/o) of PTX3. DHS sera were reconstituted with the purified human C3 protein at 5% of its serum concentration. **A)** FACS plots from a representative experiment (out of 6) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel A), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. Data are from 6 independent experiments, each performed on a different donor (6 donors in total) in duplicate. (**** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$, Student's paired t test, as described in [2]).

towards AF is complement-dependent and requires the AP, since it was abolished when human serum was heat-inactivated or depleted of FB (i.e., an essential component of the AP C3 convertase). I investigated these properties and assessed the relative contribution of CP, LP and AP using the trypan exclusion flow cytometry-based phagocytosis assay, and NHS or human sera depleted of selected complement components (DHS). Absence of serum or depletion of C3 (in C3DHS) both impaired engulfment of AF by PMNs and abolished the PTX3-dependent potentiation of phagocytosis; addition of serum or reconstitution of C3DHS with purified C3 both increased phagocytosis and rescued the pro-phagocytic activity of PTX3 (Figure 12). Therefore, functionally active C3 is required for the PTX3-dependent amplification of conidia phagocytosis, consistent with previous reports [2].

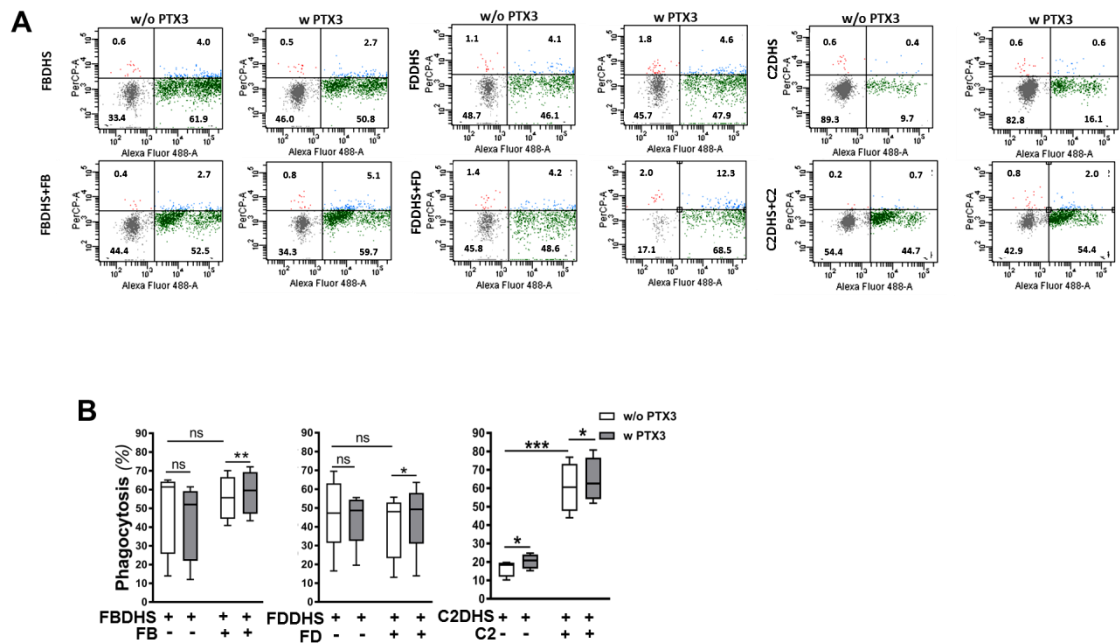


FIGURE 13. The pro-phagocytic activity of PTX3 is AP-dependent. Phagocytosis was performed as described in Figure 11 in the presence or absence of 5% NHS and human sera depleted of FB or FD (FBDHS and FDDHS, respectively; that do not make the AP C3 convertase but retain the opsonic activity of C3) and C2 (C2DHS; that do not form the CP/LP C3 convertase). DHS sera were reconstituted with the respective purified human proteins (FB, FD, C2) at 5% of their serum concentration. **A)** FACS plots from a representative experiment (out of 4 to 5) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel A), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. Data are from 4 to 5 independent experiments, each performed on a different donor (4 to 5 donors in total) in duplicate. (***P<0.001, **P<0.01, *P<0.05, Student's paired t test).

To dissect the role of the individual pathways, similar experiments were performed in the presence of human sera depleted of FB or FD (components of the AP C3 convertase; FBDHS and FDDHS, respectively) or C2 (component of the CP/LP C3 convertase; C2DHS). Depletion of FB and FD, but not C2, abolished the PTX3 effect (Figure 13), clearly indicating that it is the AP that is targeted by PTX3, despite the fact that CP/LP predominate over AP in supporting AF phagocytosis (even in the absence of PTX3) [181,334].

This was further corroborated in phagocytosis experiments where reconstituted AP (i.e., from the individual purified proteins) was used in the place of NHS. As shown in Figure 14 the percentage of FITC-positive PMNs substantially decreased (as compared to NHS) when AP proteins were applied. Moreover, PTX3 activity was retained in both conditions, clearly indicating that it is the AP (not CP neither LP) that is required for PTX3 to augment phagocytosis of AF by PMNs.

To dissect the contribution of the individual AP components to PTX3's pro-phagocytic activity, phagocytosis experiments were performed where selected proteins were removed from the AP mixtures (either FH, FB, FP, FI, FD, or C3). This revealed that AP components involved in either formation (C3 and FB), stabilization (FP) or full activation

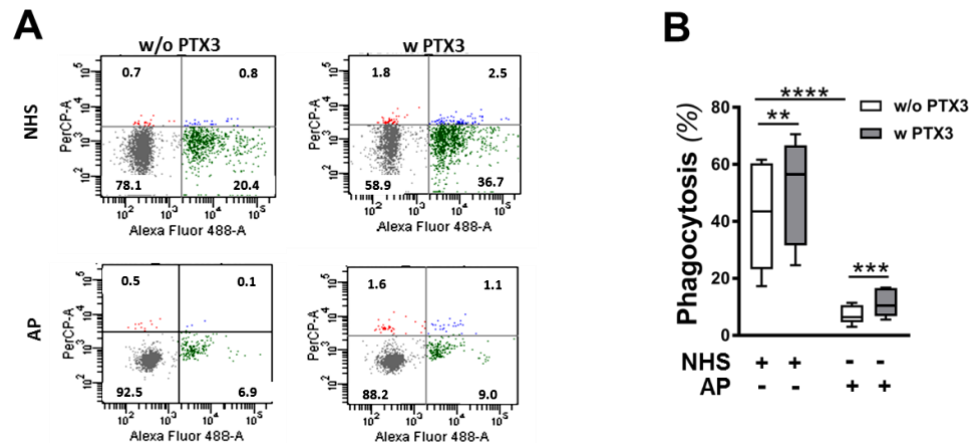


FIGURE 14. AP is sufficient to support the pro-phagocytic activity of PTX3. Effect of mixtures of purified AP proteins (reconstituted AP, at 5% of their serum concentration) on the PTX3-dependent phagocytosis of AF by PMNs. **A)** FACS plots from a representative experiment (out of 5) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel A), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. Results are from 5 independent experiments, each performed on a different in duplicate. (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, Student's paired t test).

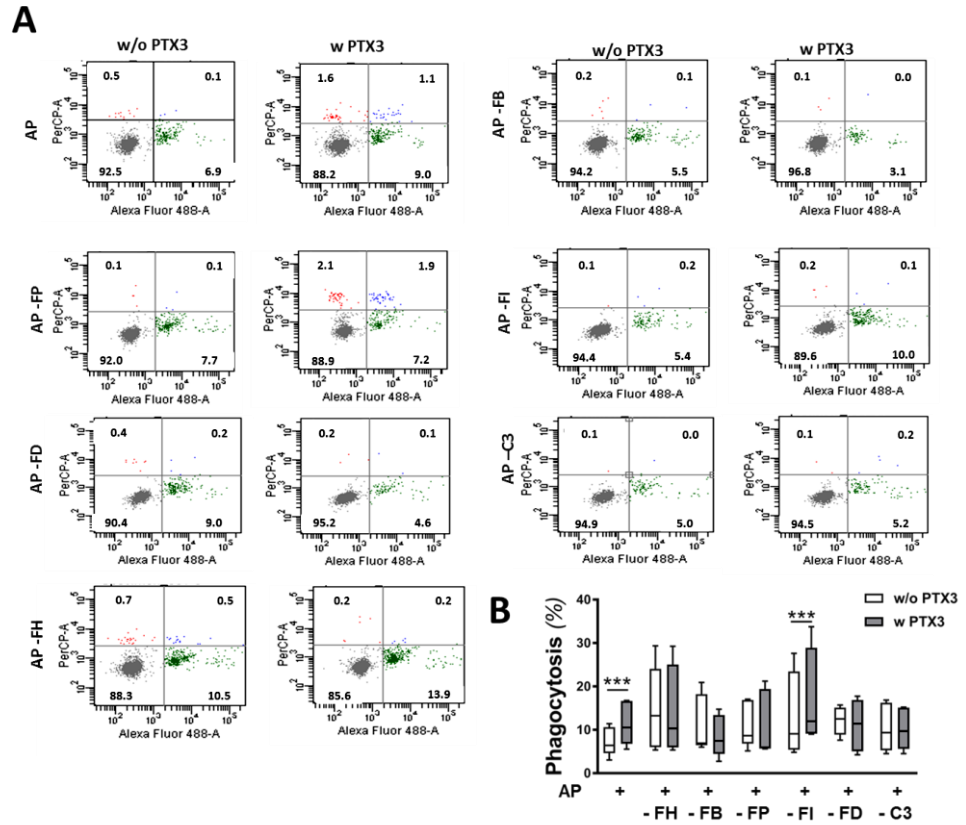


FIGURE 15. FH is required for the pro-phagocytic activity of PTX3. **A)** Phagocytosis experiments were performed as described in Figure 11 using mixtures of purified AP proteins (reconstituted AP), from which selected components (FH, FB, FP, FI, FD, or C3) were individually removed. **A)** FACS plots from a representative experiment (out of 4 to 5) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel **A**), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. Results are from 4 to 5 independent experiments, each performed on a different donor (4 to 5 donors in total) in duplicate. (***) $P < 0.001$, Student's paired t test).

(FD) of the AP C3 convertase were all required for PTX3 to promote phagocytosis of AF (Figure 15). However, when the regulators of AP (FH and FI) were individually removed, only FH was found to be necessary to support PTX3 activity, with FI playing a dispensable role in this regard.

Similar results were obtained when FH- or FI-depleted sera (FHDHS and FIDHS, respectively) were used in the place of reconstituted AP. As shown in Figure 16, PP was reduced in both conditions as compared to the corresponding supplemented sera, both in the absence and presence of PTX3. This is consistent with the notion that iC3b is a major

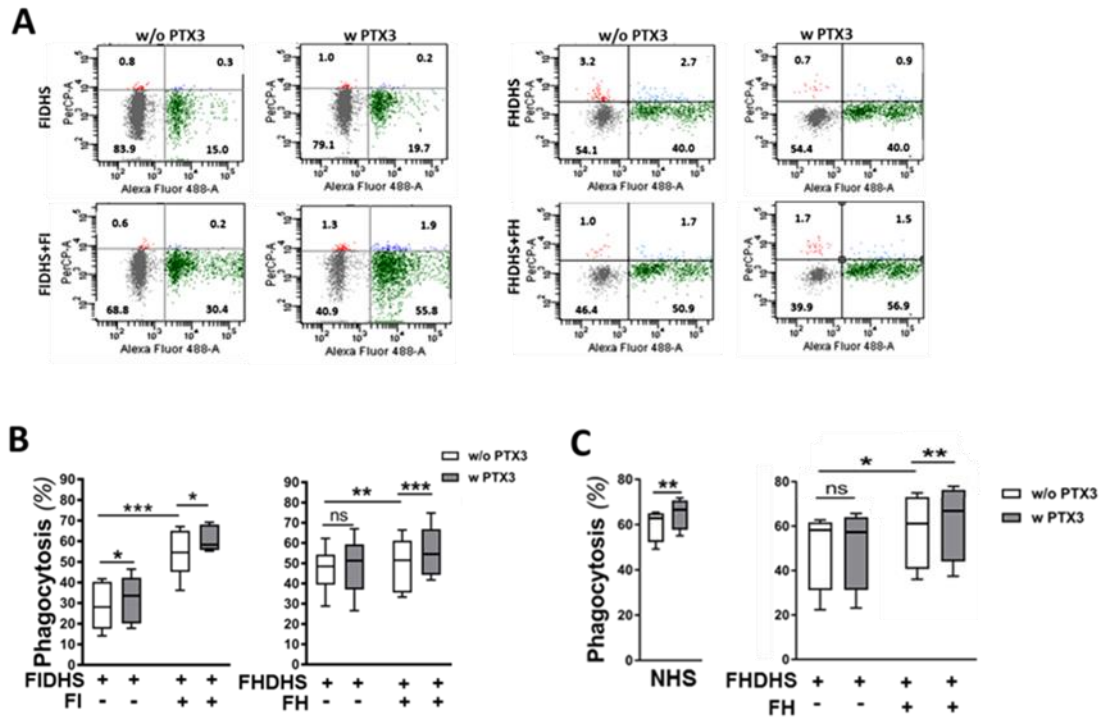


FIGURE 16. Depletion of FH from NHS abrogates the pro-phagocytic activity of PTX3. In another set of experiments, phagocytosis of AF was assessed using human sera depleted of either FI (FIDHS) or FH that had been supplemented or not with purified preparations of the missing protein. **A)** FACS plots from a representative experiment (out of 4 to 6) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel **A**), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. **C)** Similar experiments were performed using viable in the place of heat inactivated AF conidia, and NHS as a positive control. Results are expressed and reported as described in **B**. Data are from 4 to 6 independent experiments, each performed on a different donor (4 to 6 donors in total) in duplicate. (** $P < 0.001$, * $P < 0.01$, * $P < 0.05$, Student's paired t test).

complement opsonin (that is primarily recognized by CR3, see sections 1.3.1 and 1.4), and is not formed when FH or FI are missing [200].

However it is somehow in contrast with the hypothesis that FH recruitment onto the conidial wall is an evasion mechanism of AF, given that reintegration of FHDHS with purified FH enhanced rather than inhibited phagocytosis of fungal conidia by PMNs (see below, [82], and section 1.4.6). Most importantly, in FHDHS the PTX3-dependent amplification of phagocytosis was lost, and was restored upon reconstitution of FHDHS with purified FH, thus indicating that, amongst the AP components that do not directly support formation of the AP C3 convertase, FH but not FI is required for the pro-phagocytic activity of this long pentraxin. Additional *in vitro* phagocytosis experiments

were carried out using viable in the place of heat inactivated AF conidia, with similar results (Figure 16C), thus indicating that heat-inactivation of the fungal spores does not affect the pro-phagocytic activity of PTX3, as previously reported [2].

4.3 COMBINED EFFECT OF PTX3 AND FH ON KILLING OF AF CONIDIA BY HUMAN PMNS

I described that phagocytosis of AF by human PMNs is augmented by PTX3 and that this process requires FH (see Figures from 11 to 16). These findings prompted me to evaluate the effect of PTX3 on the PMN-dependent killing of fungal conidia, an effector mechanism that follows the uptake and engulfment of microbes, including AF, by phagocytic cells [335]. To this end, I set up a cell viability assay based on resazurin, an oxidation-reduction fluorogenic indicator of the mitochondrial metabolism, as described in section 3.13 of *Materials and Methods*. As shown in Figure 17A, human serum was required to promote effective elimination of fungal conidia, since in the absence of NHS, no significant changes in the MFI of resorufin (i.e., the reduced form of resazurin that is fluorescent) was observed. This highlights a tight functional cooperation between humoral and cellular (in this case, PMNs) components of the innate immune system in the recognition and killing of AF. Furthermore, PTX3 amplified the PMN-mediated killing of AF conidia both at 6 and 20h from the infectious challenge, and this occurred only in the presence of serum, indicating that other soluble pattern recognition molecules (i.e., complement) are required for the long pentraxin to promote fungal disposal. Indeed, the effect of PTX3 was abolished at both time points when FH-depleted sera (FHDHS) were used, and rescued when FHDHS was replenished with purified human FH (Figure 17B). This indicates that the crosstalk between PTX3 and the complement system, via its major soluble inhibitor FH, is important not only for phagocytosis but also killing of AF conidia by PMNs. Furthermore, supplementation of FHDHS with purified FH resulted in increased killing of AF, regardless of PTX3 (see Figure 17B). In this respect, it is worth noting that FHDHS from Complement Technology was used in this and other experiments. This serum formulation (that is supplied with 0.1 mM EDTA to inhibit spontaneous AP activation) is certified to possess functional AP when reconstituted with FH and Mg^{2+} ions, and contains normal levels of intact C3 (see www.complementtech.com for more details). The killing

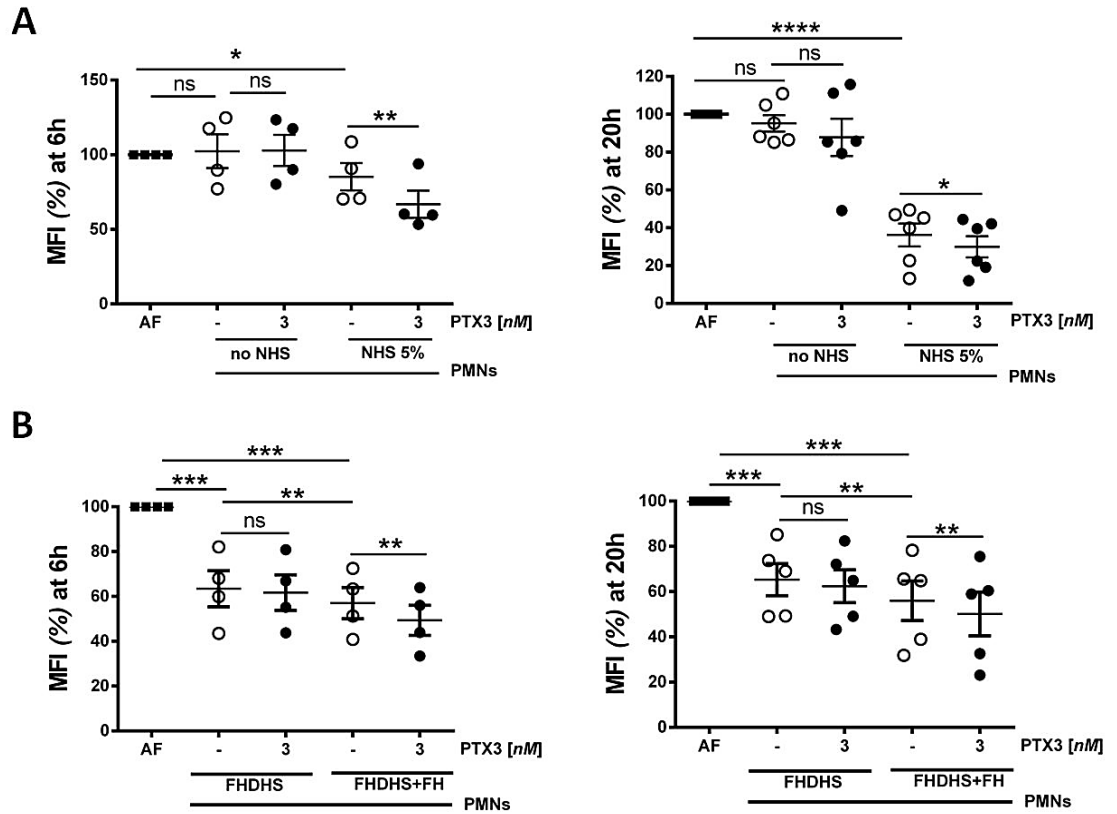


FIGURE 17. PTX3 and FH cooperate to promote killing of AF conidia by PMNs. Viable AF conidia were pre-incubated with PTX3, then further incubated with either NHS (A), FH-depleted human sera (FHDHS) or FH-supplemented FHDHS (B) in the absence and presence of freshly isolated human PMNs for 6 and 20h (left and right panels, respectively). At termination of incubation, PMNs were lysed and conidia viability was measured using Alamar Blue. Results are expressed as percentage of the resorufin fluorescence intensity recorded with no added PTX3 and PMNs (set to 100%; AF, solid squares). 4 to 6 independent experiments were performed in duplicate, each on a different donor (4 to 6 donors in total). Circles represent the mean of duplicate observations from same donor in the absence (open circles; w/o) and presence (solid circles; w) of PTX3 (overlaid bars indicate mean \pm SEM). (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, Student's paired t test).

experiments were performed in RPMI medium (that contains 0.4 mM Mg^{2+}), therefore when FH is missing extensive AP activation is expected to occur in these buffer conditions, with rapid consumption of C3 to C3b both in the fluid phase and on the conidial surface. This might explain why the percentage of viable conidia (in the absence of FH and PTX3) did not change substantially over time (median values of 62% and 63% at 6 and 20 hours from infection, respectively). However, the addition of FH promoted the PMN-dependent killing of AF conidia at both time points. This observation possibly suggests that the inhibitory activities of FH towards the AP C3 convertase do not actually spare AF conidia from complement-dependent cell-mediated killing, and rather enhance

this process perhaps via generation on the conidial surface of iC3b (that is preferentially recognized by the phagocytic receptors CR3 and CR4). This is consistent with the pro-phagocytic effect of FH described in Figure 16, and further challenges the current view that sequestration of this complement inhibitor by AF acts as an immune evasion mechanism (see [82] and section 1.4.6 of Chapter 1).

4.4 ROLE OF PTX3 IN THE ACTIVATION OF AP ON AF CONIDIA

The findings described above highlight the importance of the crosstalk between PTX3 and FH in potentiating AF phagocytosis and killing by PMNs. Given that FH regulates the stability of the AP C3 convertase (see section 1.4) in addition to inactivating it via the FI-dependent cleavage of C3b to iC3b (see [200] and Figure 18A) I evaluated if and how PTX3 could have a role in modulating deposition of the AP onto the AF conidial wall. To this end, I analysed in a time course setting AP activation and C3 fragments deposition on conidia that had been pre-incubated with PTX3 prior to challenge with reconstituted human AP. Conidia-bound C3 fragments were eluted and analysed by western blotting, using an anti-human C3 pAb that recognizes both intact and cleaved C3. As shown in Figure 18B, total C3 deposition (as monitored via the C3 β band at 75 kDa, which does not undergo proteolysis) and C3 cleavage to C3b (as followed via the C3 α' band at 105 kDa) both increased over time, as expected for AP activation. This was supported by densitometric analysis of the corresponding signals, where volumes of the C3 β band and percentages of C3b over total C3 were both augmented at the investigated time points (first and third plots, respectively, from left in Figure 18C). Neither the ratio of intact to total C3 (C3 α /C3 β) nor that of iC3b to total C3 (C3 α' 42kDa/C3 β) change over time (second and fourth plots, respectively, from left in Figure 17C), indicating that the observed accumulation of total C3 is mostly due to C3b binding to the conidial cell wall. This likely occurs via ester and/or amide covalent linkages, both hydrolysed in the alkaline NH₂OH solution used in this study to retrieve the conidia-bound C3 fragments [238]. Interestingly, pre-opsonisation of conidia with PTX3 enhanced C3b binding and inhibited iC3b formation. These effects were significant at early time points (5 and 15 minutes) and disappeared later on in the course of AP activation (30 minutes), suggesting that PTX3 acts in the early phase of this process. It is noteworthy that PTX3 did not alter the overall proteolysis of C3 (as indicated by C3 α /C3 β), neither did it modify total C3 deposition (as indicated by C3 β), indicating that it specifically acts on the relative amounts of C3b and

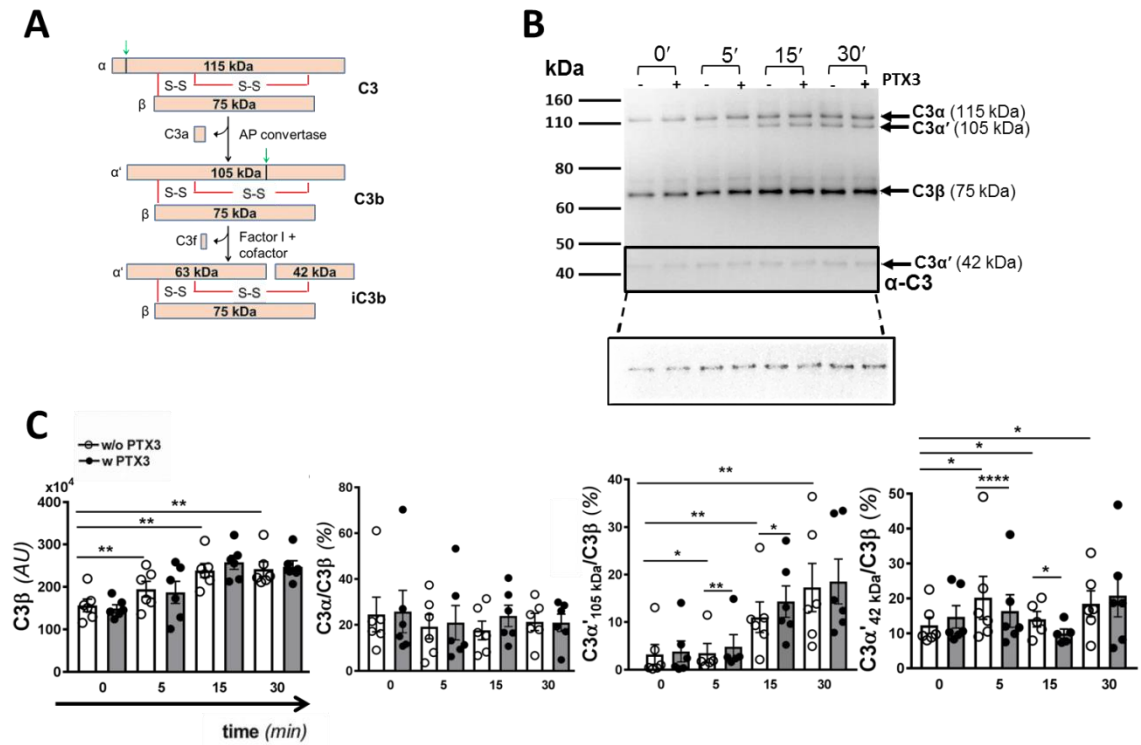


FIGURE 18. PTX3 modulates the C3b/iC3b balance on AF conidia. **A**) Schematic view of the proteolytic processing of C3 to C3b (by the AP C3 convertase) and iC3b (by FI and a cofactor, such as FH). Green arrows point to sites of proteolysis. **B**) AF conidia were pre-opsonised with a saturating concentration of PTX3 (60 nM, corresponding to 20 µg/ml, based on molecular weight of the PTX3 8-mer) or buffer, and incubated with reconstituted human AP (5% of serum concentration) for 5, 15 and 30 min. Bound C3 fragments were eluted, separated on SDS-PAGE gels, and revealed by western blotting with an anti-human C3 pAb. A representative gel is shown, with the observed C3 fragments (and the corresponding molecular weights) indicated by arrows. A lower portion of the gel (up to ~48 kDa) was expanded and contrasted for clarity. **C**) Blot images were analyzed by densitometry. Volumes of the intact C3α chain band at 115 kDa, the C3α' fragment bands at 105 and 42 kDa (i.e., that originate from proteolysis of C3α), and the C3β chain band at 75 kDa (i.e., that is not cleaved by FI) were quantitated. In the first graph from left C3β volumes (arbitrary units, AU) are plotted as representative of total C3 deposition. In the other graphs the percentage over C3β of either C3α, C3α' at 105 kDa or C3α' at 42 kDa (i.e., indicative of intact C3, C3b and iC3b, respectively) are shown. Results are from 6 independent experiments, each performed in single. Circles represent individual observations in the absence (open) and presence (closed) of PTX3. These are overlaid on bars representing mean ± SEM (**P<0.01, *P<0.05, Student's paired t test).

iC3b. These are both dependent on FH and FI [202], however FI proved dispensable for the pro-phagocytic activity of PTX3 (see Figure 16), which questions whether the observed decrease in deposition of iC3b (in the presence as compared to the absence of PTX3) is indeed involved in the PTX3-dependent promotion of AF conidia phagocytosis by PMNs. I addressed this point further in this section and in sections 4.6 to 4.9.

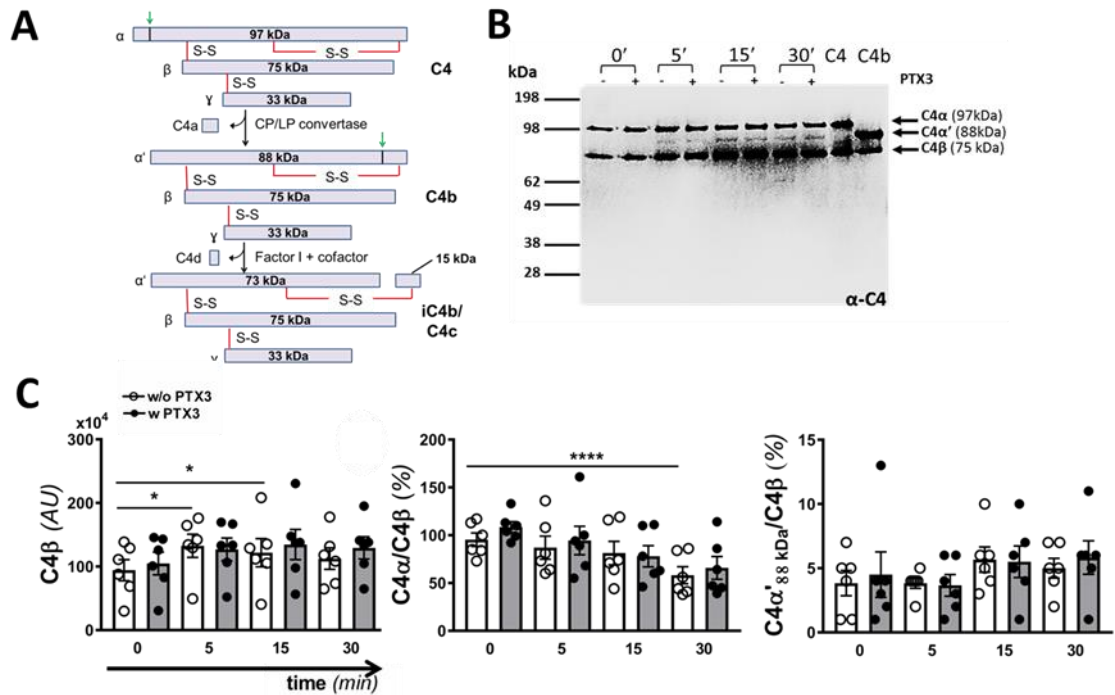


FIGURE 19. C4 deposition/teolysis on AF conidia is not affected by PTX3. **A)** Schematic view of the proteolytic processing of C4 to C4b (by the CP/LP C3 convertase) and iC4b (by FI and a cofactor, like C4BP). Green arrows point to sites of proteolysis. **B)** Deposition of C4 onto AF conidia was assessed as described in Figure 18, however 5% NHS and an anti-human C4 pAb were used in the place of reconstituted AP and anti-human C3 pAb, respectively. Purified human C4 and C4b were run as controls. A representative gel is shown, with the observed C4 fragments (and the corresponding molecular weights) indicated by arrows. **C)** Volumes of the intact C4 α chain band at 97 kDa, the C4 α' fragment band at 88 kDa (i.e., that originates from proteolysis of C4 α), and the C4 β chain band at 75 kDa (i.e., that is not cleaved by FI) were measured. In the first graph from left C4 β volumes (arbitrary units, AU) are plotted as representative of total C4 deposition. In the other graphs the percentage over C4 β of either C4 α or C4 α' at 88 kDa (i.e., indicative of intact C4 and C4b, respectively) are shown. Results are from 6 independent experiments, each performed in single. Circles represent individual observations in the absence (open) and presence (closed) of PTX3. These are overlaid on bars representing mean \pm SEM (**** P <0.0001, * P <0.05, Student's paired t test).

These findings indicate that PTX3 acts on AP and regulates the C3b/iC3b balance on the AF conidial surface. However, I could not rule out an effect of PTX3 on C4 deposition too, although my (Figure 13) and previous [181] observations indicate that CP and LP are not required for the pro-phagocytic activity of PTX3. It was important to address this point given that CP and LP are increasingly recognized as prominent pathways of complement activation on AF [181,334]. Therefore, in similar experimental conditions to those described for AP (see Figure 18), I analysed deposition and proteolysis (see scheme in Figure 19A) of C4 on AF conidia, using normal human serum (NHS) as a source of C4 and

an appropriate anti-human C4 pAb. Figures 19B and C indicate that total C4 deposition (as monitored via the C4 β band at 75 kDa, which, like C3 β , does not undergo proteolysis in the applied conditions) increased at early time points (5 and 15 min), and net processing of C4 (as indicated by diminishing percentages of intact over total C4, C4 α /C4 β) increased over time (with a statistical significance at 30 min), consistent with CP/LP activation. However, the C4b fraction (C4 α '88kDa/C4 β plot in Figure 19C) did not change significantly over time, possibly due to mixed contributions of C4 and C4b binding to conidia. I could not track iC4b formation in the applied experimental conditions, since the C4 pAb did not detect the corresponding C4 α ' bands at 73 and 15 kDa, likely due to poor affinity of the antibody for these fragments. Most interestingly, pre-treatment of conidia with PTX3 did not modify the relative amounts of total and intact C4, neither did it alter the percentage of deposited C4b at any time (Figure 19C), suggesting that PTX3 does not affect formation and proteolytic processing of the CP/LP C3 convertase on AF conidia.

C3b and iC3b both act as opsonins that promote recognition and phagocytosis of microbes, including AF, by PMNs [336-338], and their relative abundance on microbial cells is controlled by the proteolytic activity of FI in conjunction with co-factors, most notably FH (see Figure 18A and [339]). Based on the observation that FH is required for the pro-phagocytic and pro-killing activities of PTX3 (see Figures 16 and 17), and that this long pentraxin “tips” the balance between C3b and iC3b on AF conidia (i.e., by enhancing the binding of C3b and inhibiting that of iC3b, see Figure 18B and C), I questioned whether FH and/or FI are involved in this process. To this end, complement activation on AF conidia was assessed as described in Figure 18, however human sera depleted of either FH or FI (FH- and FIDHS, respectively) were used in the place of NHS. In this case, C3 fragments were analysed by western blotting after 5 minutes incubation only, when the effect of PTX3 on C3b deposition was maximal (see Figure 18B and C). Gel images and the corresponding density plots in Figure 20A and B indicate that removal of FH abolished the PTX3-dependent enhancement of C3b deposition, and this activity was rescued upon reconstitution of FHDHS with purified FH (Figure 20B). FI had no effect on PTX3, since this retained its pro-C3b potential regardless of the protease (Figure 19A). As expected, depletion of either FH or FI caused a marked increase in bound C3b, consistent with the notion that both factors are required for effective proteolysis of C3b to iC3b [339]. In line with this, iC3b formation could not be detected in this assay (i.e., in the absence of either complement protein).

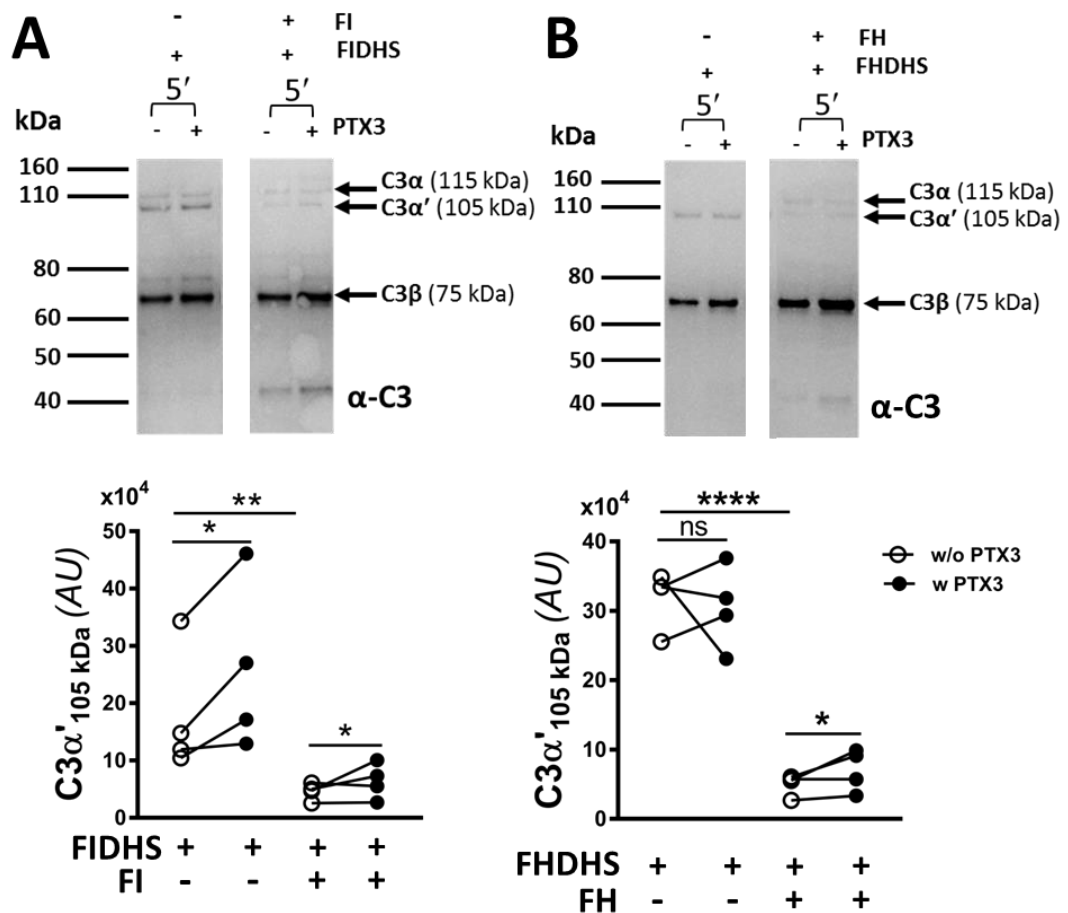


FIGURE 20. FH is required for PTX3 to enhance opsonisation of AF conidia by C3b. The combined effect of PTX3 and FI (A) or FH (B) on C3 deposition onto AF conidia was investigated using 5% FHDHS or FIDHS and FH- or FI-supplemented FHDHS/FIDHS. C3 fragments were revealed by western blotting after 5 min incubation; representative gels are shown in the upper panels. Blot images were analyzed by densitometry, and volumes (arbitrary units, AU) of the observed C3 fragment bands were measured. Plots of the volumes of the C3α' fragment band at 105 kDa (i.e., indicative of C3b) as a function of treatments are reported in the lower panels. Circles represent individual observations in the absence (open) and in the presence (closed) of PTX3. (4 independent experiments performed in single; ****P<0.0001, **P<0.01, *P<0.05, Student's paired t test).

4.5 ANALYSIS OF THE INTERACTION OF PTX3 AND FH WITH AF CONIDIA

These findings point to FH as a key contributor of the pro-phagocytic and pro-killing properties of PTX3 (see above). FH is a ligand of AF conidia [246], and PTX3 recognizes sites on this protein (i.e., CCP modules 7 and 19-20) that have been reported to mediate binding to the fungal spores [315]. Therefore, I hypothesized that this long pentraxin might affect the interaction of AF with FH, and in this way modulate C3b and iC3b

formation/deposition on the conidial wall. To test this hypothesis, I first characterized the interaction with AF of the individual proteins, then I assessed if and how they affect each other's binding to fungal conidia. Heat-inactivated fungal conidia were incubated with recombinant human PTX3 (3-60 nM, based on MW of the PTX3 8-mer), and bound protein was revealed with a biotinylated α -human PTX3 pAb by flow cytometry. As shown in Figure 21A, PTX3 bound AF conidia in a dose-dependent fashion with saturating and half maximal concentrations of ~ 30 and ~ 6 nM, respectively. I next identified the PTX3 domain/s (see Figure 21B) that mediate/s recognition of AF. Flow cytometry analyses performed with the α -human PTX3 pAb (that recognizes both N_PTX3 and C_PTX3) indicated that most, if not all, of the PTX3 binding to AF is mediated by the protein's N-terminal domain (Figure 21C); C_PTX3 had poor, if any, interaction with conidia, even when applied at 60 nM_{eq} (i.e., twice the saturating concentration of PTX3, based on the notion that C_PTX3 forms monomers with a MW of ~ 25 kDa and eight C-terminal domains are present in the full length PTX3 protein; $[C_PTX3]_{1\text{-mer}} = 8 \times [PTX3]_{8\text{-mer}}$ [298]). To further corroborate this observation, I analysed the interaction of PTX3, N_PTX3 and C_PTX3 with AF conidia using an alternative experimental approach, based on microtitre filter plates and monoclonal antibodies that specifically recognize epitopes of the N- and C-terminal regions of the protein. In this case, both PTX3 and N_PTX3 were found to dose-dependently interact with AF and saturate binding sites on fungal conidia at similar molar equivalent concentrations (15-30 nM_{eq}, based on the notion that N_PTX3 forms 4-mers with a MW of ~ 75 kDa and two N-terminal 4-mers are present in the full length PTX3 protein; $[N_PTX3]_{4\text{-mer}} = 2 \times [PTX3]_{8\text{mer}}$ [298]) (Figure 20D, upper panel). No binding was observed for C_PTX3 at any applied concentration, even using a monoclonal antibody that specifically targets this domain (Figure 21D, lower panel). Furthermore, the binding profiles observed for PTX3 using the α -N_PTX3 and α -C_PTX3 monoclonal antibodies (upper and lower panels of Figure 21D, respectively) appeared different, likely due to diverse affinities of the two antibodies for the full length protein (of which they recognize distinct epitopes in the N- and C-terminal domains, [340]. Nonetheless, in equilibrium conditions, like in the FACS (Figure 21C) and ELISA (Figure 21D) setups used in these experiments, C_PTX3 did not show any interaction with AF conidia. To rule out artefacts potentially arising from non-specific interactions, a preparation of human CRP (from plasma), which has been reported to lack recognition of resting AF conidia [341], was used in ELISA experiments. Despite of CRP and PTX3 sharing common

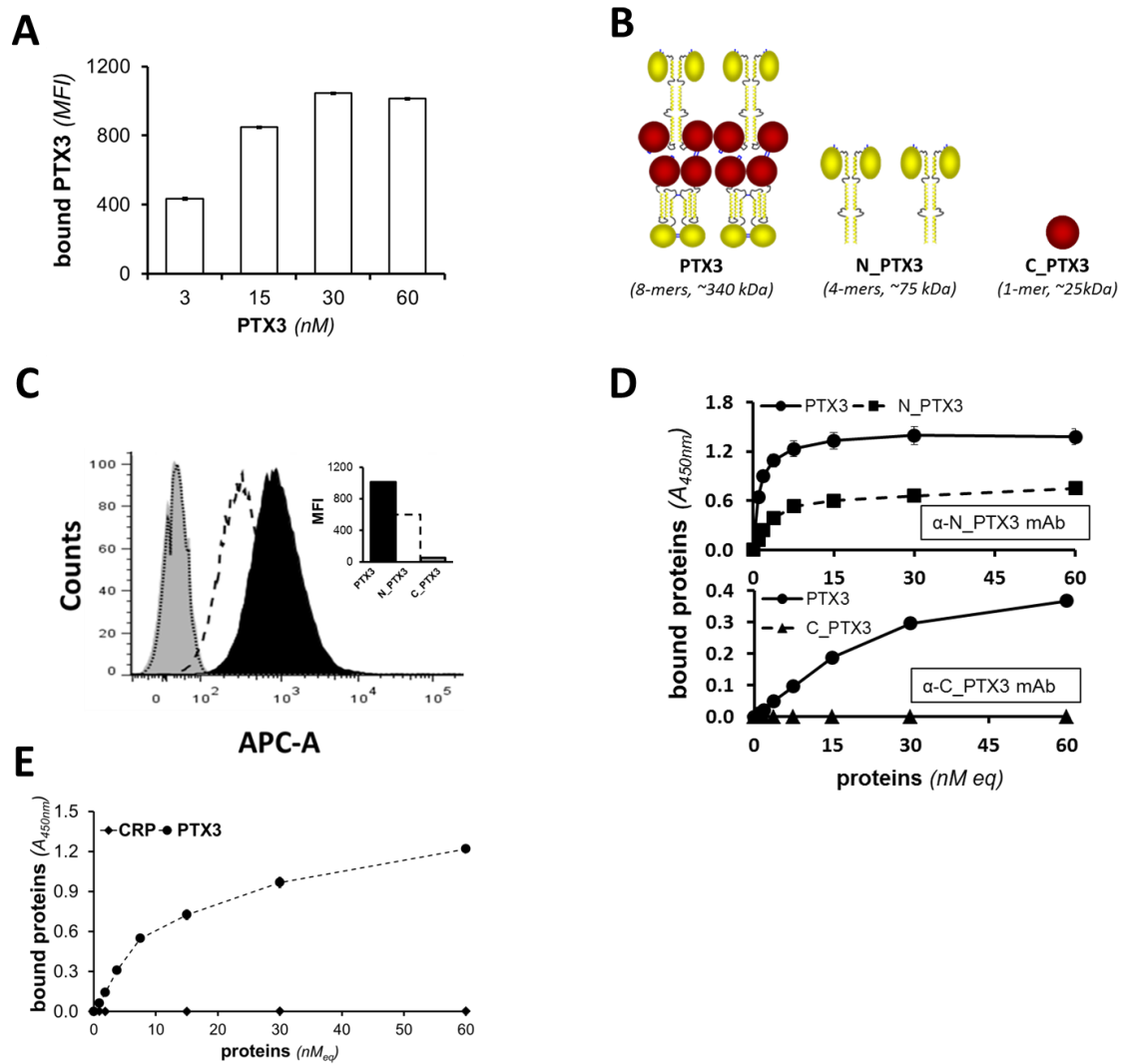


FIGURE 21. PTX3 recognizes AF conidia via its N-terminal domain. **A)** Flow cytometry analysis of the PTX3 binding to AF conidia as assessed using a biotinylated α -human PTX3 pAb. Results are expressed as MFI (3 experiments done in duplicate; $n=6$, mean \pm SEM). **B)** Schematics of the full-length PTX3 protein and two recombinant constructs comprising its N- (in yellow) and C- (in red) terminal domains (N_PTX3 and C_PTX3, respectively). Oligomer state and molecular weight of each protein are indicated. **C)** Binding of PTX3, N_PTX3 and C_PTX3 (all at 60 nM_{eq}; as defined in the main text) was measured by flow cytometry as described in **B**. A plot of event counts vs fluorescence intensity, representative of 3 independent experiments performed in duplicate, is shown. In the inset is a bar graph displaying the MFI values recorded for the three proteins, generated with data from all experiments ($n=6$, mean \pm SEM). **D)** Conidia were incubated in microtitre filter plates with the indicated concentrations of PTX3, N_PTX3 and C_PTX3, and bound proteins were revealed with mAbs raised against either the N- or the C-terminal domain (upper and lower panel, respectively) (3 experiments done in duplicate; $n=6$, mean \pm SEM). **E)** Conidia were incubated in microtitre filter plates with the indicated concentrations of PTX3 and CRP, and bound proteins were revealed as described in *Materials and Methods* (2 experiments done in quadruplicate; $n=8$, mean \pm SEM).

structural features (i.e., the pentraxin domain), this short pentraxin failed to bind the fungal

spores at any applied concentration (Figure 21E), strongly indicating that the N-terminal region of PTX3 (not its C-terminal domain) contains genuine binding sites for AF.

To further characterize the interaction of AF conidia with PTX3 and its isolated domains, in collaboration with Attana AB (Stockholm, Sweden), I developed a new application of the Attana's quartz crystal microbalance (QCM) technology (see scheme in Figure 22A). In this regard, QCM biosensors have proved to be a powerful tool for label-free and real-time analysis of biomolecular interactions under dynamic conditions, enabling the study of binding kinetics. Furthermore, QCM cell biosensors have been recently developed where intact cells are grown or captured on polystyrene-coated QCM chips (cell compatible sensor chips), thus allowing the study of biomolecular interactions on cell surfaces, which more closely mimics the natural biological environment [342,343].

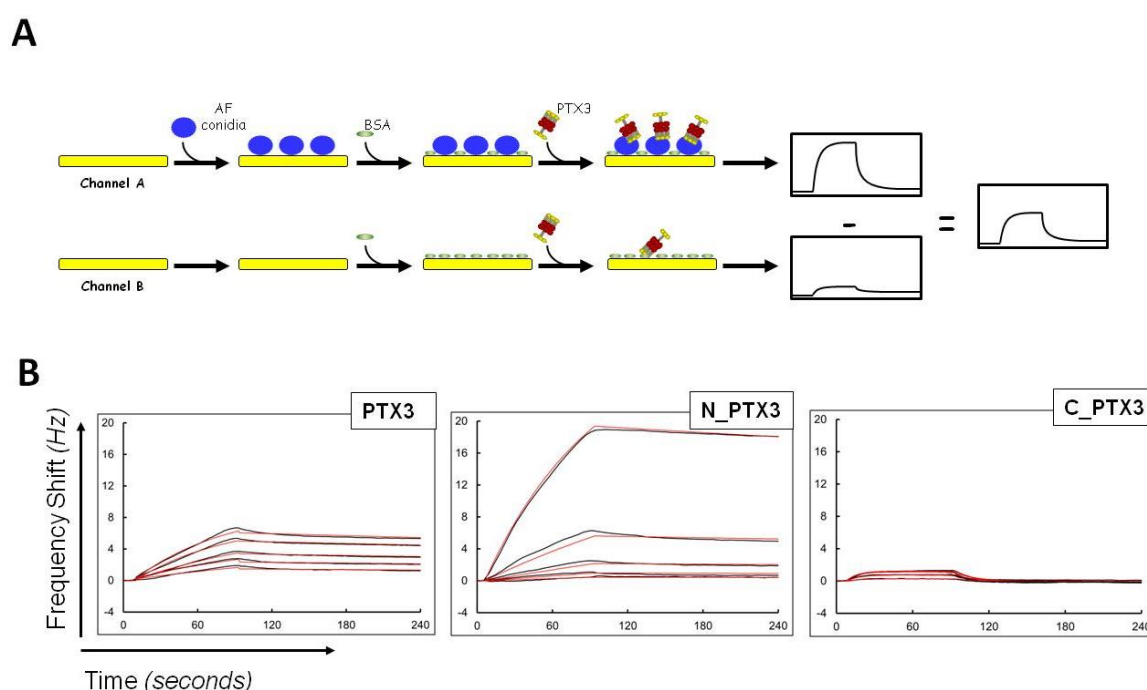


FIGURE 22. Kinetic analysis of the interaction with AF conidia of PTX3 and its domains. **A)** Schematic of the experimental strategy. AF conidia were adsorbed onto QCM cell-compatible sensor chips, and free sites were blocked with BSA (channel A). A control chip was made that contained BSA only (channel B). Following baseline stabilization, PTX3 proteins (only full-length PTX3 is shown for clarity) were injected and QCM frequency shift (in Hz) was recorded as a function of time (s). Sensorgrams were reference- and buffer-subtracted prior to kinetic fitting. **B)** Representative sensorgrams from one out of two independent experiments (black lines) are shown, where either PTX3 (1 in 2 dilution series), N_PTX3 (1 in 3 dilution series) or C_PTX3 (1 in 2 dilution series) were injected onto AF-coated chips at a range of protein concentrations (0.8 to 80 nM_{eq}). Fitting curves (overlaid red lines) were generated by global fitting of the recorded sensorgrams to a 1:1 Langmuir binding model.

In the following experiments, AF conidia were adsorbed onto cell compatible sensor chips and PTX3 proteins were injected at a range of concentrations (Figure 22B). Global fitting of the recorded sensorgrams to a 1:1 Langmuir binding model allowed measurement of both the kinetic rate constants k_{on} and k_{off} , and, indirectly (i.e., from the k_{off}/k_{on} ratio), the equilibrium dissociation constant K_D of the individual protein/AF complexes. As shown in Table 2, conidia were recognized by both PTX3 and N_PTX3 with high affinity (K_D values of 0.669 ± 0.001 nM and 3.72 ± 0.12 nM, respectively). The apparent difference in K_D amongst the two proteins was mostly due to the association kinetics (k_{on} values of $1.08 \times 10^6 \pm 7.42 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ and $1.32 \times 10^5 \pm 22.2 \text{ M}^{-1} \text{ s}^{-1}$ for PTX3 and N_PTX3, respectively), whereas both dissociated from AF conidia at comparable rates (k_{off} values of $7.25 \times 10^{-4} \pm 7.19 \times 10^{-7} \text{ s}^{-1}$ and $4.90 \times 10^{-4} \pm 1.55 \times 10^{-5} \text{ s}^{-1}$ for PTX3 and N_PTX3, respectively). This indicates that PTX3 binds AF with a faster kinetics than N_PTX3, however both proteins form similarly stable complexes with AF. Interestingly, the measured K_D value for the

TABLE 2. Rate and equilibrium constants from QCM analysis of the interaction with AF conidia of the PTX3 proteins and FH. Values are from two independent multi cycle kinetic experiments.

Protein	Rate and equilibrium constants (mean \pm SE)		
	k_{on} (1/M·s)	k_{off} (1/s)	K_D (nM)
PTX3	$1.08 \times 10^6 \pm 7.42 \times 10^2$	$7.25 \times 10^{-4} \pm 7.19 \times 10^{-7}$	0.669 \pm0.001
PTX3-2S	$4.08 \times 10^5 \pm 24.7$	$7.57 \times 10^{-4} \pm 1.44 \times 10^{-5}$	1.85 \pm0.04
PTX3-5S	$1.58 \times 10^5 \pm 33.1$	$1.66 \times 10^{-4} \pm 3.37 \times 10^{-5}$	1.05 \pm0.21
N_PTX3	$1.33 \times 10^5 \pm 22.2$	$4.90 \times 10^{-4} \pm 1.55 \times 10^{-5}$	3.72 \pm0.12
N_PTX3-3S	$2.20 \times 10^5 \pm 1.10 \times 10^2$	$6.29 \times 10^{-4} \pm 1.41 \times 10^{-5}$	2.86 \pm0.07
C_PTX3	$2.29 \times 10^5 \pm 91.5$	$7.42 \times 10^{-2} \pm 7.02 \times 10^{-5}$	324 \pm3
FH	$1.63 \times 10^5 \pm 19.5$	$6.51 \times 10^{-4} \pm 6.43 \times 10^{-6}$	3.99 \pm0.04

PTX3-AF interaction (0.669 ± 0.001 nM) is consistent with the observation that as low concentrations as 0.6 nM are sufficient for PTX3 to promote AF phagocytosis by PMNs *in vitro* (see Figure 11), and suggests that this long pentraxin might retain its pro-phagocytic activity (at least *in vitro*) at even lower concentrations than 0.6 nM. As compared to PTX3

and N_PTX3, C_PTX3 had a poorer affinity for AF conidia (K_D value of $324 \pm 3 \text{ nM}$), with a faster dissociation rate (k_{off} value of $7.42 \times 10^{-2} \pm 7.02 \times 10^{-5} \text{ s}^{-1}$) than those of both PTX3 and N_PTX3, and an association rate (k_{on}) of $2.29 \times 10^5 \pm 91.5 \text{ M}^{-1} \text{ s}^{-1}$, similar to that of N_PTX3, however lower than that of PTX3 (see Table 2). This suggests formation of unstable, dissociation-prone AF/C_PTX3 complexes, which might explain why this interaction was not clearly seen when assessed under equilibrium conditions (see Figure 21C and D).

Overall, binding experiments performed under both equilibrium (i.e., by flow cytometry and ELISA) and dynamic (i.e., by QCM) conditions indicate that AF binding is largely mediated by the N-terminal domain of PTX3, consistent with previous reports [2]. In addition, based on QCM data, it is conceivable that association of the full length PTX3 protein with AF conidia (k_{on}) involves sites in both protein domains, which might explain why k_{on} decreased (compared to that of the full length PTX3) when the C-terminal domain was missing (i.e., in the N_PTX3 construct; see Table 2). It is worth noting here that the frequency shifts observed for N_PTX3 were larger than those recorded for PTX3, which is surprising given the MW of the two proteins (~ 75 vs ~ 340 kDa). This might be ascribed to different (i.e., PTX3 vs N_PTX3) water displacement effects (that contribute to the frequency signal recorded in QCM, as described in the “Materials and Methods” section), or, alternatively, to deeper penetration of N_PTX3 (as compared to the parent protein) into the cell wall of AF conidia (where the PTX3 ligand/s is/are presumably located), likely due to the smaller size of this domain. In this regard higher density of binding sites has been observed for N_PTX3 (as compared to the intact full length PTX3) in preliminary single molecule atomic force microscopy analyses, where either N_PTX3 or PTX3 were used as probes to scan the cell wall of AF conidia (Inforzato et al, personal communication). Furthermore, to rule out the possibility that the high frequency shifts observed for N_PTX3 in QCM (see middle panel of Figure 22B) originated from protein aggregates in the applied N_PTX3 preparations, these (all at 1 mg/ml , corresponding to $6 \mu\text{M}_{\text{eq}}$, a concentration well above the highest used in QCM analyses, i.e. $80 \text{ nM}_{\text{eq}}$) were chromatographed on a Superdex 200 SEC column. The representative chromatogram in Figure 23A clearly shows that no high molecular weight aggregates were present in the N_PTX3 solutions. In addition, SDS-PAGE analyses indicated that under non-reducing conditions, N_PTX3 is composed of two species with estimated masses of 74 and 35 kDa (Figure 23B, left panel), and migrates as a single band of 18 kDa under reducing conditions (right panel), consistent with the theoretical molecular mass of this domain ($18,163 \text{ Da}$, as calculated from the

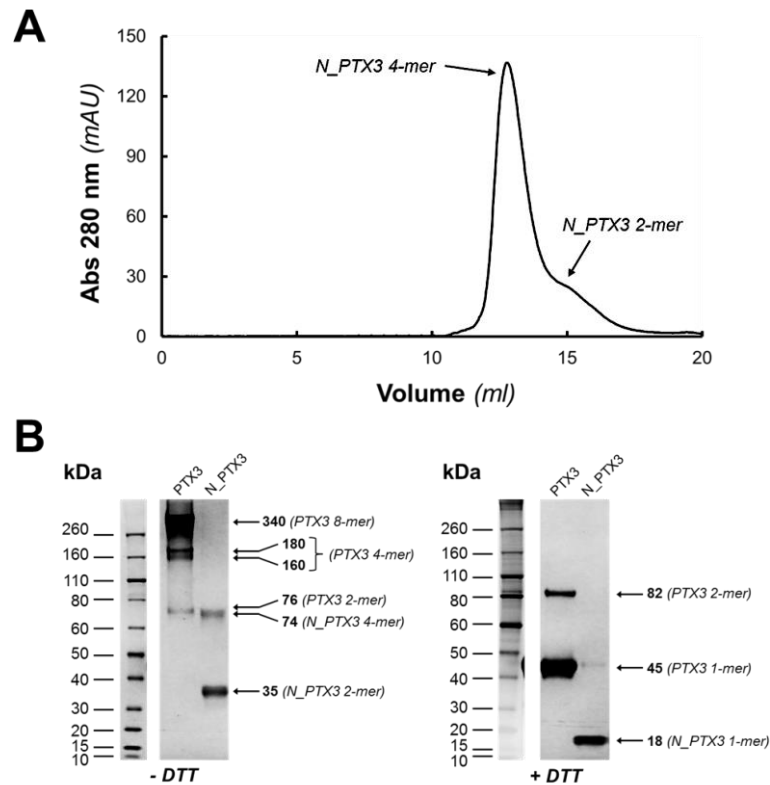


FIGURE 23. SEC and SDS-PAGE analysis of N_PTX3. **A)** 1 mg/ml solutions of N_PTX3 in PBS⁺ were chromatographed on a Superdex 200 GL 10/300 column using an ÄKTA Purifier system, equilibrated and eluted with PBS⁺ at 0.5 ml/min. Protein separation was monitored by UV absorbance at 280 nm. Shown is a representative chromatogram (from 3 independent experiments), indicating that N_PTX3 forms predominantly tetramers (4-mers), with a minor (approximately 10%) fraction of total protein present as dimers (2-mer). **B)** N_PTX3 (and PTX3) were run under denaturing conditions on 4–12% (w/v) Bis-Tris gels, in the absence (- DTT) and presence (+DTT) of dithiothreitol. Following separation, protein bands were stained with Bio-Safe™ Coomassie (Bio-Rad). Representative gels from 3 independent experiments are shown, with molecular mass markers on the left, and apparent molecular mass values observed for the resolved bands on the right.

protein sequence). Thus, the 74- and 35-kDa species correspond to disulfide bond-linked tetramers and dimers of N_PTX3, respectively, as previously described [297]. In the applied experimental conditions, PTX3 (used as a control for DTT reduction) generates a pattern of bands that is consistent with previous reports [298].

I extended my investigations to the interaction of FH with AF conidia, using both equilibrium and dynamic settings. A dose-dependent interaction of AF conidia with this complement component was observed using microtitre filter plate binding assays, with saturating and half maximal concentrations of ~67 and ~9 nM, respectively (Figure 24A). Furthermore, QCM analyses of the FH binding to AF-coated sensor chips were performed in the same experimental conditions as described for PTX3 and its domains (Figure 22B). In this case, values of $1.63 \times 10^5 \pm 19.5 \text{ M}^{-1} \text{ s}^{-1}$ and $6.51 \times 10^{-4} \pm 6.43 \times 10^{-6} \text{ s}^{-1}$ were measured for k_{on} and k_{off} , respectively, which resulted in a K_D of $3.99 \pm 0.04 \text{ nM}$. This is ~6 times higher than that observed for PTX3 (i.e., $0.669 \pm 0.001 \text{ nM}$), a difference that could be

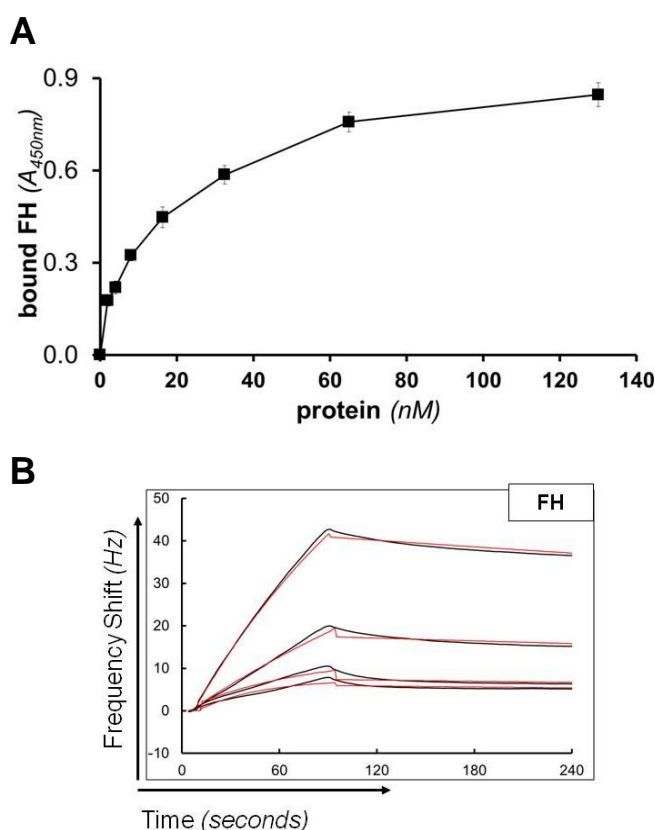


FIGURE 24. Characterization of the interaction of FH with AF conidia. **A)** FH binding to AF conidia was assessed using microtitre filter plates as described in *Materials and Methods* (3 experiments done in quadruplicate; $n=12$, mean \pm SEM). **B)** Kinetics of the AF-FH interaction was investigated using the QCM strategy described in Figure 17. FH was injected onto AF-coated chips at a range of protein concentrations (20 to 300 nM, 1 to 2 dilution series), and QCM frequency (in Hz) was recorded as a function of time (s). Representative sensorgrams from one out of two independent experiment (black lines) are shown, where fitting curves (overlaid red lines) were generated by global fitting of the recorded sensorgrams to a 1:1 Langmuir binding model.

mostly ascribed to slower association (as compared to PTX3, see Table 2). These findings are consistent with previous reports on the binding of FH to AF conidia [82], moreover they provide novel insights into the kinetic properties of this interaction.

4.6 EFFECT OF PTX3 ON THE INTERACTION OF FH WITH AF CONIDIA

Following up on my investigations on the interaction with AF of the individual PTX3 and FH proteins, I assessed if and how these affect each other's binding to fungal conidia. To

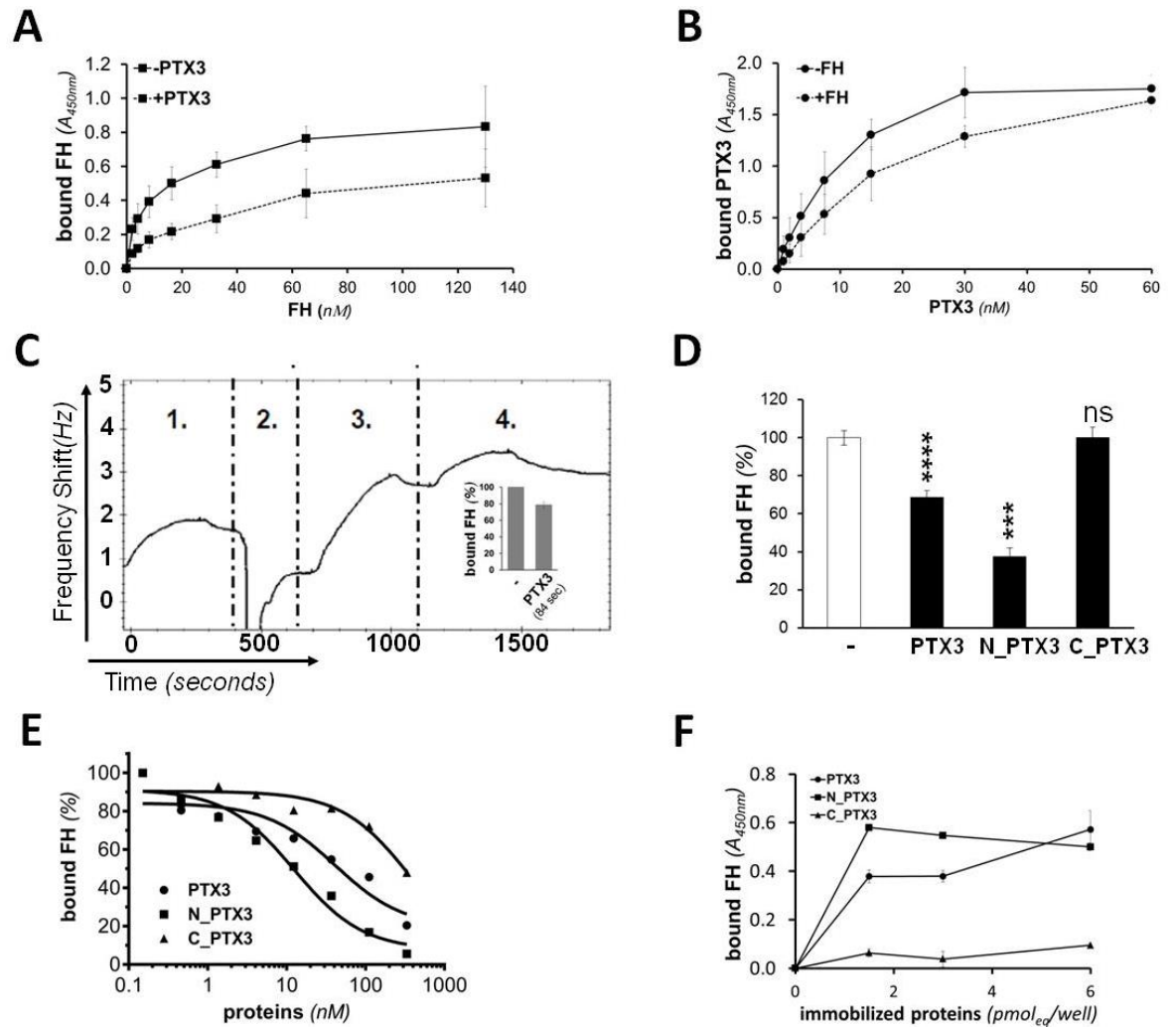


FIGURE 25. PTX3 shares with FH common binding sites on AF conidia. **A** and **B**) Binding of FH (**A**) and PTX3 (**B**) to AF conidia was assessed using microtitre filter plates, following pre-incubation of plates with PTX3 (**A**) and FH (**B**) or buffer (-PTX3 or -FH in either panel). Bound proteins were revealed with the corresponding polyclonal antibodies, and results are expressed as absorbance values at 450 nm (2 experiments done in triplicate; $n=6$, mean \pm SEM). **C**) The effect of PTX3 pre-opsonisation on FH binding to AF conidia was investigated using the QCM approach described in Figure 22. Briefly, FH was injected onto AF-coated chips at 50 nM (1), chip surface was regenerated using 22 mM formic acid, pH 2.50 (2), and PTX3 was applied for 84s at 60 nM (3) prior to an additional injection of FH (4). Frequency shifts (in Hz) were recorded under steady state conditions in 1 and 4, and the percentage of residual FH binding (after injection of PTX3) was calculated from the ratio of the frequency response in 4 compared to 1 (inset). Representative results from two independent experiments are shown (mean \pm SEM in the inset). **D**) The effect of PTX3 domains on FH binding to AF conidia was evaluated in sequential incubation experiments performed with microtitre plates. Conidia were pre-incubated with either PTX3, N_PTX3 or C_PTX3 (all at 60nM_{eq}), prior to addition of FH (133nM), and bound FH was revealed as described in **A**. Results from 3 experiments done in quadruplicate are reported as percentage of FH binding in the absence of the PTX3 proteins ($n=12$, mean \pm SEM; **** $P<0.0001$, *** $P<0.001$, compared to no added PTX3 proteins, Student's t test). *Legend to figure continues on next page.*

Legend to figure continues from previous page. E) In co-incubation experiments, conidia were incubated in microtitre plates with 3.3 nM of FH and a range of PTX3, N_PTX3 or C_PTX3 concentrations (0.45-333 nM_{eq}). Bound FH was revealed as described in **A**, and results are expressed as percentage of FH binding (2 experiments done in quadruplicate; n=8, mean \pm SEM). Experimental values (symbols) were fitted to a one-site competition model using the GraphPad Prism software. Shown are the best fitting curves (solid lines; $R^2 > 0.85$). **F)** Binding of FH (at 17 nM) to microtitre plates coated with the indicated amounts of PTX3, N_PTX3 or C_PTX3 was assessed by ELISA. Results are shown as absorbance values at 450 nm (2 experiments done in duplicate; n=4, mean \pm SEM).

this end, sequential incubation experiments were performed using microtitre filter plates, where AF conidia that had been pre-incubated with either protein at concentrations above binding saturation (60 and 130 nM for PTX3 and FH, respectively) were further incubated with the other interactant. As shown in Figure 25A, pre-opsonisation of AF conidia with PTX3 inhibited the binding of FH at any applied concentration. Furthermore, when AF conidia were pre-incubated with FH, the binding of PTX3 also was inhibited at all concentrations (Figure 25B), however the inhibitory effect of FH towards PTX3 was smaller than that observed for PTX3 towards FH (~89.2% vs ~66.3% of PTX3 and FH residual binding at the highest applied concentration of FH and PTX3, respectively). These findings suggest that PTX3 and FH share common binding sites on the AF conidial surface, thus possibly acting as mutual competitive inhibitors; moreover, the difference observed in the inhibitory activity is consistent with QCM indicating that PTX3 is a stronger binder of AF than FH (see Table 2). To rule out antibody effects (possibly arising from pre-incubation of AF conidia with one protein affecting structure and thereby affinity of the other for its corresponding antibody) we took advantage of the antibody-free QCM strategy described in Figure 22A, and performed sequential injection experiments. As shown in Figure 25C, pre-injection (for 84s) of PTX3 over AF-coated sensor chips caused a decrease in FH binding, as measured under steady state conditions ($78.6 \pm 3.8\%$ of residual FH binding); furthermore, when PTX3 was pre-injected for longer times (300 s, which resulted in additional binding to AF), a stronger inhibitory effect was observed ($66.2 \pm 7.6\%$ of residual FH binding; data not shown). This is consistent with results from experiments performed under equilibrium conditions (Figure 25A) and support the hypothesis that PTX3 and FH recognize common or, at least, partially overlapping binding sites on AF conidia. To further substantiate this point, additional experiments were performed using N_PTX3 and C_PTX3, whose AF binding characteristics have been described above (see Figures 21C and D, 22B, and Table 2). In microtitre plates, pre-

opsonisation with either PTX3 or N_PTX3 caused a significant inhibition of FH binding to fungal conidia, and N_PTX3 proved to be a more potent inhibitor than PTX3 (38% *vs* 69% of residual FH binding; Figure 25D). C_PTX3 had no effect on the association of AF with the FH, in agreement with our observation that this domain has a minor, if any, role in the PTX3-AF interaction (Figures 21C and D, and 22B). These findings indicate that the N-terminal domain of PTX3 recapitulates the inhibitory activity of the full-length protein towards FH. To further address this point, co-incubation experiments were performed, where FH was incubated with a range of concentrations of the PTX3 proteins prior to addition of conidia. As expected, PTX3 and N_PTX3 inhibited FH binding to AF in a dose-dependent fashion; however, C_PTX3 had only a minor effect (Figure 25E). In all circumstances, N_PTX3 was a stronger inhibitor than PTX3 (IC_{50} values of 12.5 *vs* 41.4 nM_{eq}), possibly due to N_PTX3 occupying more binding sites than PTX3 on the conidial surface (as suggested by QCM, see Figure 22B). Furthermore, the inhibitory effect of both PTX3 and N_PTX3 was stronger than that observed in sequential incubation experiments (Figure 25D), likely due to PTX3 and N_PTX3 sequestering FH in solution in addition to acting as competitive inhibitors. Consistent with this, FH preferentially bound the N-terminal domain of PTX3 (Figure 25F). However, FH had a minor, although not negligible, interaction with the C-terminal region of PTX3, which might explain the mild inhibitory activity of C_PTX3 observed in co-incubation experiments (Figure 25E). In this respect, it is known that CCP7 and CCPs 19-20 of FH are recognized by the C- and N-terminal domains of PTX3, respectively [315].

C4BP, co-factor of FI in the proteolytic processing of C4 and major soluble inhibitor of the CP/LP C3 convertase [344], is recruited onto the AF conidial wall, where this is regarded as an additional strategy to escape complement-mediated immune reactions [246,82]. In the light of my findings on the PTX3-FH interplay on AF conidia (see Figure 25), and given that PTX3 recognizes sites on the C4BP protein (i.e., α chain) that have been implicated in the recognition of AF conidia [4], I assessed whether this long pentraxin also affects the interaction of AF with C4BP. As shown in Figure 26A, a dose-dependent interaction of C4BP with AF conidia was observed in microtitre binding assays. Moreover, in sequential incubation experiments, pre-opsonisation with either PTX3 or N_PTX3 caused a significant inhibition of C4BP binding to fungal conidia, and N_PTX3 proved to be a more potent inhibitor than PTX3 (71% *vs* 86% of residual C4BP binding; Figure 26B). C_PTX3 had no effect on the association of AF with this complement protein. These findings suggest that PTX3, C4BP (and FH) likely share common binding sites on AF

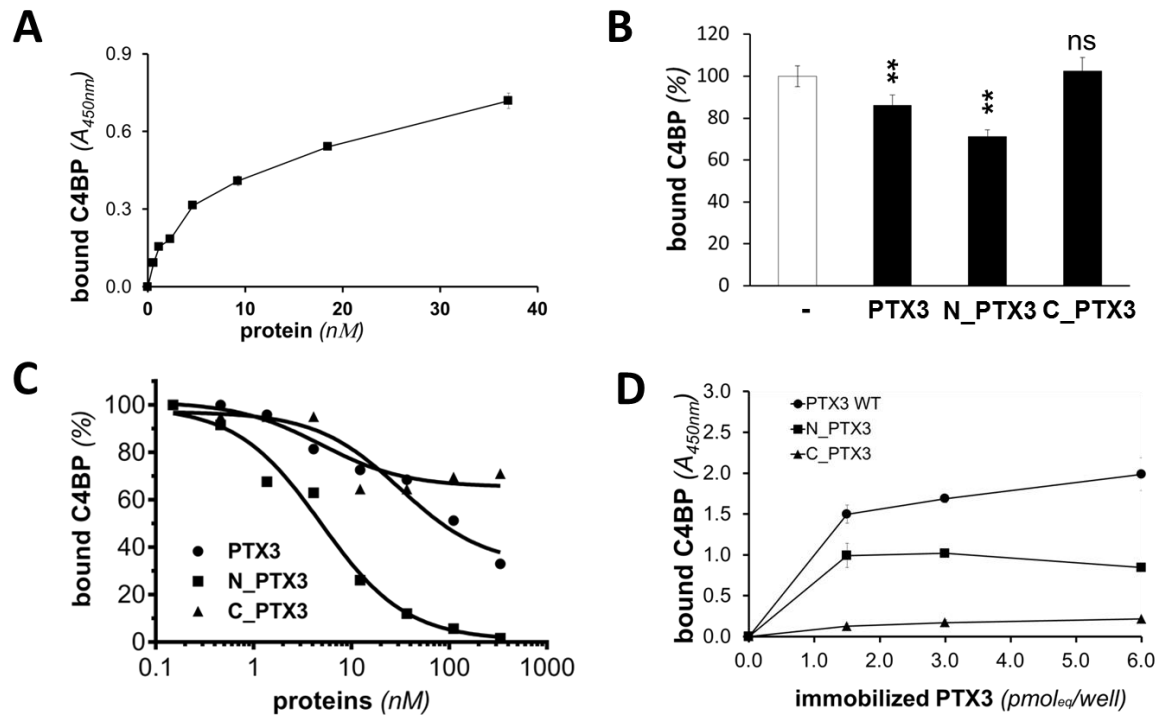


FIGURE 26. PTX3 shares with C4BP common binding sites on AF conidia. **A)** C4BP binding to AF conidia was assessed using microtitre filter plates as described in section 3.2 (2 experiments done in quadruplicate; $n=8$, mean \pm SEM). **B)** In sequential incubation experiments, conidia were pre-incubated with either PTX3, N_PTX3 or C_PTX3 (all at 60 nM_{eq}), prior to addition of C4BP (35 nM). Bound C4BP was detected with a polyclonal antibody, and results were expressed as percentage of C4BP binding in the absence of the PTX3 proteins (3 experiments done in quadruplicate; $n=12$, mean \pm SEM; ** $P<0.01$ compared to no added PTX3 proteins, Student's t test). **C)** In co-incubation experiments, conidia were incubated with 3.3 nM of C4BP and a range of PTX3, N_PTX3 and C_PTX3 concentrations (0.45-333 nM_{eq}). Bound C4BP was revealed as described in **A**, and results are expressed as percentage of free C4BP binding (3 experiments done in quadruplicate; $n=12$, mean \pm SEM). Experimental values (symbols) were fitted to a one-site competition model using the GraphPad Prism software. Shown are the best fitting curves (solid lines; $R^2 > 0.85$). **D)** Binding of C4BP (at 17 nM) to microtitre plates coated with the indicated amounts of PTX3, N_PTX3 or C_PTX3 was assessed by ELISA. Results are shown as absorbance values at 450 nm (2 experiments done in duplicate; $n=4$, mean \pm SEM).

conidia, and PTX3, via its N-terminal domain, can act as a competitive inhibitor of C4BP (and FH). To further evaluate this activity, co-incubation experiments were performed, similar to those described for FH (see Figure 25E). Here, PTX3 and N_PTX3 both inhibited C4BP binding to AF in a dose-dependent fashion; C_PTX3 instead had only a minor effect (Figure 26C). Again, N_PTX3 was a stronger inhibitor than PTX3 (IC₅₀ values of 4.9 vs 83.2 nM_{eq}), and the inhibitory effect of both proteins was more pronounced than that observed in sequential incubation experiments (Figure 26B). Consistent with this, C4BP preferentially bound the N-terminal domain of PTX3 (Figure

26D), similar to what is described for FH (Figure 25F). However, a minor, although not negligible, interaction with the C-terminal region of PTX3 was detected, which might explain the mild inhibitory activity of C_PTX3 observed in co-incubation experiments (Figure 26C). It is worth mentioning here that I provided the first evidence that the N-terminal region of PTX3 mediates most of the protein's binding to C4BP, whereas the C-terminal domain has a less important role in this interaction. Furthermore, it is worth pointing out that all ELISA experiments were performed using buffers containing BSA (to suppress nonspecific interactions), and all QCM runs were carried out on chip-immobilized conidia that had been blocked with BSA prior to injections of the analytes (see the "Materials and Methods" chapter for more experimental details).

4.7 EFFECT OF PTX3 ON THE COFACTOR AND AP C3 CONVERTASE INHIBITING/DECAY-ACCELERATING ACTIVITIES OF FH ON AF CONIDIA

As a soluble inhibitor of AP, FH acts as a cofactor in the FI-mediated cleavage of C3b to iC3b (see Figure 18A and section 1.4.5). To assess the effect of PTX3 and its domains on this activity, fungal conidia were sequentially incubated with either PTX3, N_PTX3 or C_PTX3 and FH, followed by addition of a mixture of C3b and FI. The proteolytic reactions were monitored by western blotting using a polyclonal antibody that recognizes intact C3b (α' and β chains) as well as the C3 α' fragments at 63 and 42 kDa, which are diagnostic of iC3b (Figure 27A, upper panel). When conidia were pre-incubated with PTX3 or N_PTX3, the relative concentration of these fragments was significantly reduced, as quantitatively assessed by densitometry (Figure 27A, lower panel). Furthermore, the N-terminal domain was more active than the full-length protein (Figure 27A). C_PTX3 did not affect the FI-mediated cleavage of C3b, as iC3b fragment bands had the same intensity as in control experiments (i.e., without PTX3 proteins). These results are consistent with the observations that PTX3 and N_PTX3, but not C_PTX3, inhibit binding of FH to AF conidia (see Figure 25D and E), and indicate that this inhibitory effect impacts on the cofactor activity of the regulator, ultimately leading to a reduced proteolysis of C3b.

As an additional mechanism of complement inhibition, FH can both impair formation of the AP C3 convertase and accelerate dissociation of this complex into its composing subunits (see section 1.4.5 and [200]). To evaluate the effect of PTX3 on these activities

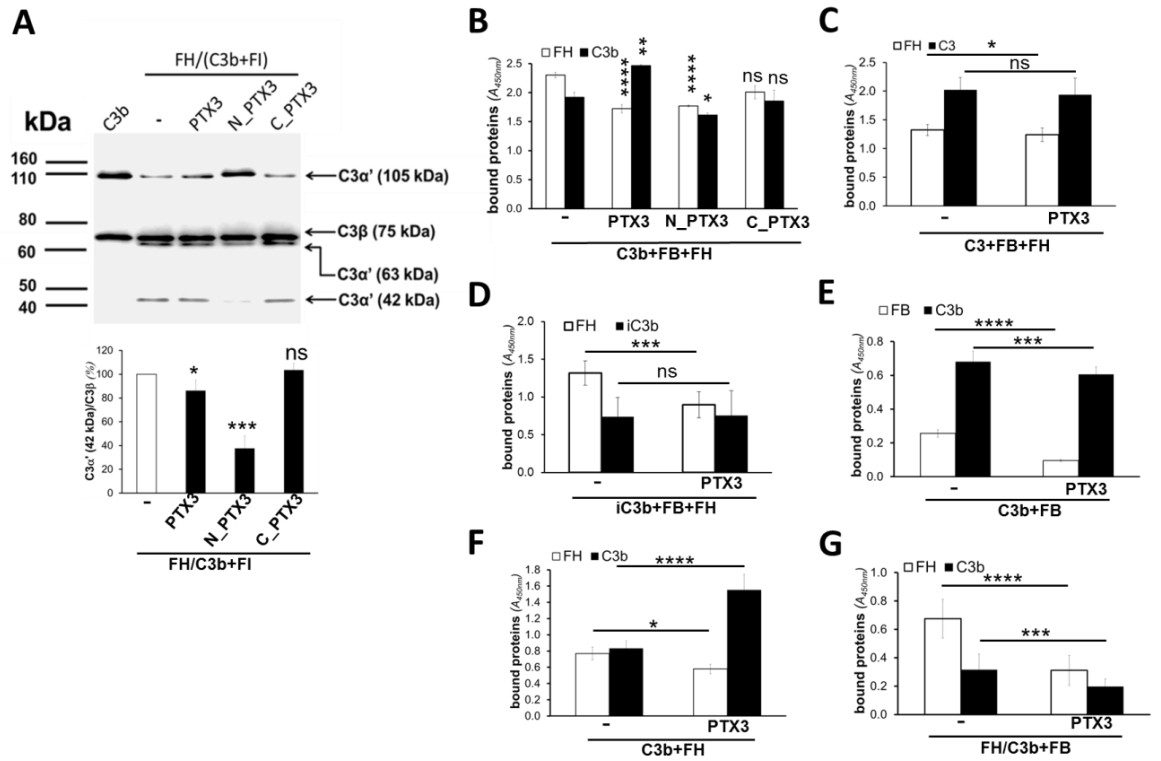


FIGURE 27. PTX3 inhibits the cofactor activity of FH and recruits C3b onto AF conidia in the presence of FH. **A)** AF conidia were pre-incubated with either PTX3, N_PTX3 or C_PTX3, prior to addition of FH. Unbound proteins were removed, and a mixture of C3b and FI was applied. Reaction products (i.e., C3 fragments) were revealed by western blotting using an α -C3 pAb. Experiments performed in the absence of the PTX3 proteins (-) or FI (C3b) were taken as positive and negative controls, respectively (a gel is shown in the upper panel that is representative of 4 independent experiments). Blot images were analyzed by densitometry. Intensities of the C3 α' fragment band at 42 kDa (i.e., that originates from proteolysis of the C3 α' chain) and the C3 β chain band at 75 kDa (i.e., that is not cleaved by FI and can be used as a normalization factor) were measured in the absence (open box) and in the presence (solid boxes) of the PTX3 proteins (lower panel). Results are expressed as percentage of the C3 α' (42 kDa)/C3 β volume ratio in the absence of the PTX3 proteins (4 experiments performed in single; n=4, mean \pm SEM; ***P<0.001, *P<0.1 compared to no added PTX3 proteins; Student's t test). **B-D)** AF conidia (1×10^7 /well) were pre-opsonised in microtitre filter plates with PTX3 (or N_PTX3 and C_PTX3 in **B**), then incubated with FB and FH in the presence of either C3b (**B**), C3 (**C**) or iC3b (**D**). Bound FH and C3 proteins were revealed with α -FH and α -C3 pAbs, and results were expressed as absorbance at 450 nm (n=6 from 3 independent experiments performed in duplicate; mean \pm SEM; ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05, Student's t test). **E-G)** In similar experiments, AF conidia were pre-opsonised with PTX3, then incubated with mixtures containing either C3b and FB (**E**) or C3b and FH (**F**). In another set of experiments, plates were incubated with FH prior to addition of a mixture of C3b and FB (**G**). Bound proteins were revealed with α -FH, α -C3 or α -FB pAbs, and results are expressed as absorbance at 450 nm (n=4 from 2 independent experiments performed in duplicate; mean \pm SEM; ****P<0.0001, ***P<0.001, *P<0.05, Student's t test).

we performed a series of microtitre plate binding experiments, where AF conidia that had been pre-incubated with PTX3 (or, in some experiments, N_PTX3 and C_PTX3) were further incubated with selected combinations of C3b, FB and FH. When the complement proteins were applied together (C3b+FB+FH), PTX3 pre-opsonisation led to a small but significant increase in the deposition of C3b (Figure 27B). This cannot be ascribed to formation on the conidial surface of a PTX3/FH/C3b ternary complex (whose occurrence in solid phase binding experiments has been previously documented [315]), since PTX3 concomitantly inhibited FH binding to AF conidia. Furthermore, pre-incubation of AF conidia with N_PTX3 resulted in inhibition rather than promotion of the binding of C3b, and no effect was observed for C_PTX3 (Figure 27B). The PTX3-dependent recruitment onto AF conidia of C3 fragments was restricted to C3b, since pre-opsonisation of fungal conidia with the long pentraxin had no effect on intact C3 and inactivated C3b (iC3b), when these proteins were used in the place of C3b (Figure 27C and D, respectively). To assess whether PTX3 had a role in formation/stabilization of the AP C3 convertase on AF conidia (possibly as a consequence of the FH binding to AF being inhibited by PTX3), I performed similar experiments, however using different combinations of proteins and incubation conditions. In particular, when mixtures of C3b and FB (essential components of the AP C3 convertase) were applied, pre-opsonisation of AF conidia with PTX3 did not increase, but rather inhibited the interaction of C3b with AF (Figure 27E). However, when mixtures of C3b and FH were incubated with AF conidia, the amount of bound C3b increased significantly following pre-opsonisation of PTX3 (as compared to no added PTX3), similar to the condition where C3b, FH and FB were all present (Figure 27F and, for comparison, Figure 27B). Furthermore, in experiments where AF conidia were sequentially incubated with PTX3, FH and mixtures of C3b and FB, PTX3 appeared to inhibit C3b binding (Figure 27G), analogous to what is shown in Figure 27E. These findings suggest that PTX3 can selectively (amongst the C3 fragments) recruit C3b onto AF conidia via a FH-dependent mechanism that does not require FB (or C3 convertase formation/stabilization), and FH needs to encounter C3b in solution in order for C3b to interact with the conidia-bound PTX3.

To further investigate the FH-dependent interaction of C3b with PTX3 in the fluid phase, I exploited a size exclusion chromatography (SEC) strategy, as described in section 3.7. Different combinations of purified PTX3, FH and C3b proteins were separated on two Superose 6 HR 10/30 SEC columns mounted in series on an FPLC chromatography system, where fractions were collected and assessed by western blotting. As shown in Figure 28A and B, respectively, PTX3 did not form a stable complex in solution with either C3b or FH, when the corresponding binary mixtures were chromatographed, despite the fact that PTX3 does interact with FH on solid surfaces [315]. Complex formation was observed, instead, when FH was pre-incubated with C3b prior to SEC separation (red box

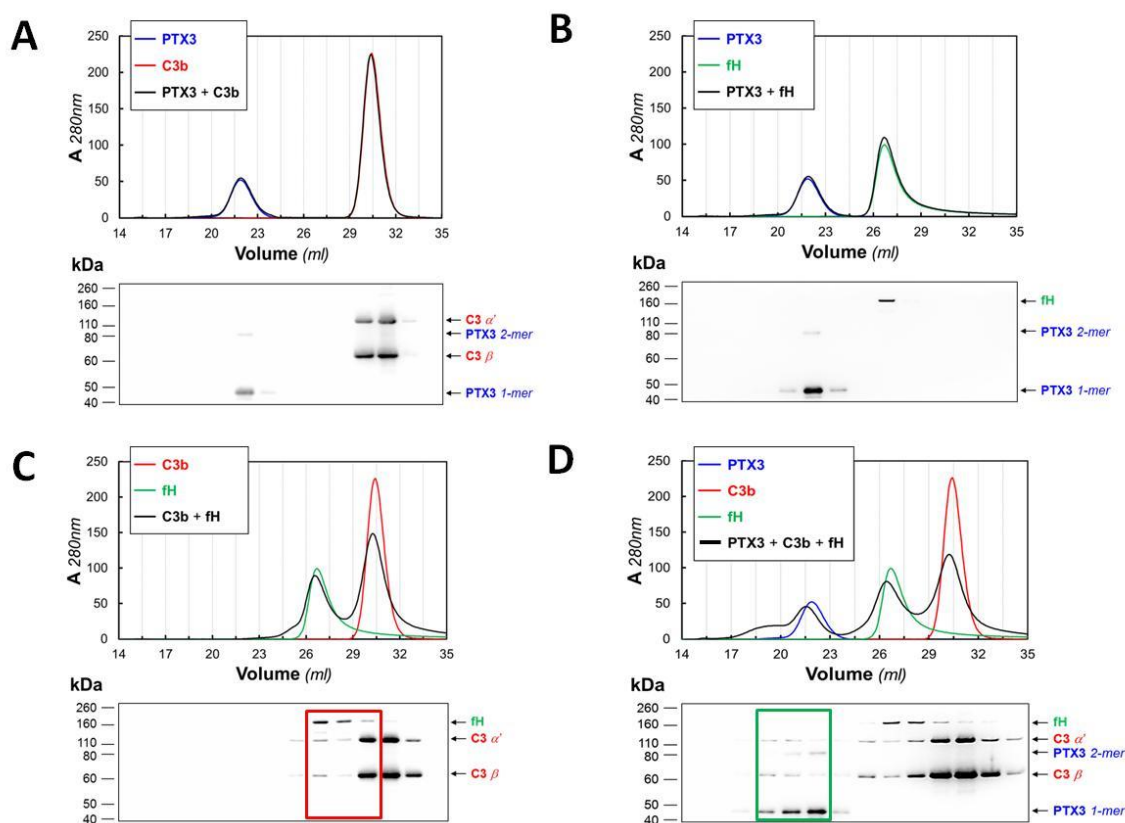


FIGURE 28. PTX3 binds C3b in solution in a FH-dependent manner. PTX3 was incubated with either C3b, FH or a mixture of both molecules (PTX3+C3b in **A**, PTX3+FH in **B** and PTX3+C3b+FH in **D**). As a control, C3b was incubated with FH (**C**). Protein mixtures were separated on two Superose 6 HR 10/30 SEC columns connected in series, and chromatograms were recorded as UV absorbance at 280 nm (black lines in the upper panels). The individual proteins were run under identical conditions (blue, red and green lines in the upper panels). 1.5 ml fractions (whose boundaries are marked by dotted grey lines in the chromatograms) were collected and analyzed by Western Blotting, using α -PTX3, α -FH and α -C3 pAbs (lower panels). Representative results from two independent experiments are shown. Red (panel C) and green (panel D) boxes are reported to highlight multi-molecular complexes (i.e., with two protein components detected in the same SEC fraction).

on the gel in Figure 28C), consistent with the notion that C3b is a ligand of FH [345]. Most importantly, high molecular weight complexes were isolated when ternary mixtures made of C3b, FH and PTX3 were chromatographed, however these complexes contain PTX3 and C3b only, with no evidence (within the sensitivity limit of the applied western blotting procedure) of FH (green box on the gel in Figure 28D). Based on these observations and the findings described above (see Figure 27), I speculate that PTX3 interacts with C3b in the presence of FH forming a transient ternary complex, which readily loses FH and turns into a stable binary PTX3/C3b complex (that is able to bind AF conidia). The proposed mechanism requires that C3b be primed by FH in solution for binding to PTX3; the resulting PTX3/C3b complex is then recruited to the conidial surface. In other words, I hypothesise that when in a complex with FH, C3b exposes/gains binding sites for PTX3, which mediate its recruitment (via binding to PTX3) to the conidial surface.

4.8 EFFECT OF PTX3 ON THE COFACTOR AND CP/LP C3 CONVERTASE INHIBITING/DECAY-ACCELERATING ACTIVITIES OF C4BP ON AF CONIDIA

PTX3, via its N-terminal domain, inhibits the binding of C4BP to AF conidia (see Figure 26). As a major soluble inhibitor of the CP and LP, C4BP acts as a cofactor in the FI-mediated cleavage of C4b to iC4b (see Figure 19A). To assess the effect of PTX3 and its domains on this activity, fungal conidia were sequentially incubated with either PTX3, N_PTX3 or C_PTX3 and C4BP, followed by addition of a mixture of C4b and FI, similar to what described for FH (see Figure 27). The proteolytic reactions were monitored by western blotting using a polyclonal antibody that recognizes intact C4b (α' , β and γ chains), as well as the C4 α' fragment at 15 kDa, which is diagnostic of iC4b (Figure 29A). When conidia were pre-incubated with PTX3 or N_PTX3, the relative concentration of the iC4b fragment was reduced, as quantitatively assessed by densitometry (Figure 29B). C_PTX3 did not affect the C4BP- and FI-mediated cleavage of C4b, as the iC4b band had the same intensity as in control experiments (i.e., without PTX3 proteins). In addition, by adaptation of a previous protocol [4] that combines mAbs raised against intact C4b and all deposited C4b fragments (α -C4c and α -C4d, respectively) to assess the C4BP- and FI-mediated cleavage of C4b, I showed that both PTX3 and N_PTX3 inhibited C4b breakdown, with no effect of C_PTX3 (Figure 29C). These results are consistent with our observation that PTX3 and N_PTX3, but not C_PTX3, inhibit the binding of C4BP to AF

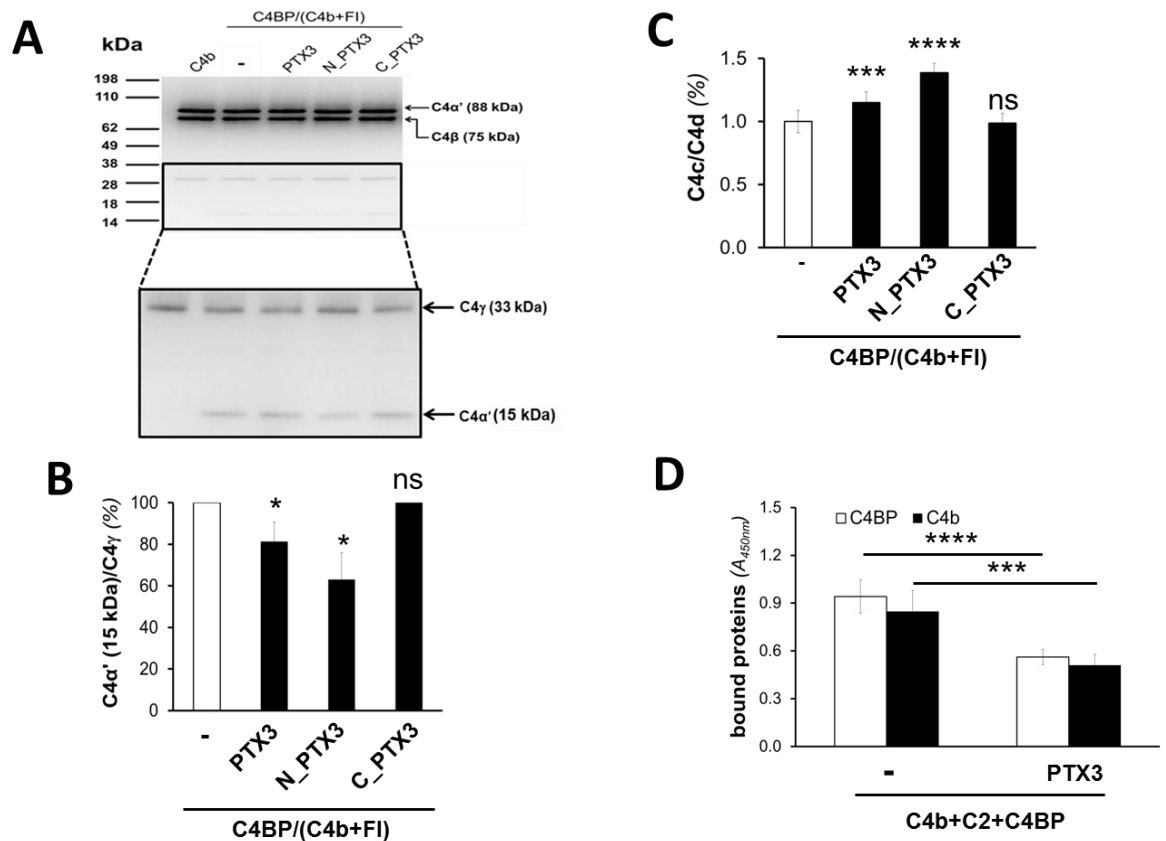


FIGURE 29. PTX3 inhibits the cofactor activity of C4BP but does not recruit C4b onto AF conidia. **A)** AF conidia were pre-incubated with either PTX3, N_PTX3 or C_PTX3, prior to addition of C4BP. Unbound proteins were removed, and a mixture of C4b and FI was applied. Reaction products (i.e., C4 fragments) were revealed by western blotting using an α -C4 pAb. Experiments performed in the absence of the PTX3 proteins (-) or FI (C4b) were taken as positive and negative controls, respectively. The lower portion (up to ~38 kDa) of a gel (representative of 3 independent experiments) was expanded and contrasted for clarity. **B)** Blot images were analyzed by densitometry. Volumes of the C4 α' fragment band at 15 kDa (i.e., that originates from proteolysis of the C4 α' chain) and the C4 γ chain band at 33 kDa (i.e., that is not cleaved by FI and can be used as a normalization factor) were measured in the absence (open box) and in the presence (solid boxes) of the PTX3 proteins. Results are expressed as percentage of the C4 α' (15 kDa)/C4 γ volume ratio in the absence of the PTX3 proteins (3 experiments performed; n=3, mean \pm SEM; *P<0.1 compared to no added PTX3 proteins; Student's t test). **C)** At termination of proteolysis, conidia were transferred onto filter plates and bound C4c and C4d were revealed with the corresponding mAbs. The anti-C4c mAb only detects intact C4b (i.e., upon C4b cleavage, C4c will not remain surface bound), and the anti-C4d mAb detects all deposited C4b fragments (i.e., both intact and cleaved C4b), since the C4d portion of C4b remains conidia-bound [4]. Ratios of the absorbance from individual mAbs were measured (C4c/C4d), and results are expressed as percentage of the C4c/C4d value in the absence of the PTX3 proteins (3 experiments done in triplicate; n=9, mean \pm SEM; ****P<0.0001, ***P<0.001 compared to no added PTX3 proteins; Student's t test). **D)** AF conidia were sequentially incubated with PTX3 and mixtures of C4b, C2 and C4BP. Bound C4BP and C4b were revealed with the corresponding pAbs. Results are expressed as absorbance values at 450 nm (2 independent experiments performed with 8 replicates; n=16, mean \pm SEM; ****P<0.0001, ***P<0.001, Student's t test).

conidia (see Figure 26B and C), and indicate that this inhibitory effect impacts on the cofactor activity of the complement regulator, ultimately leading to reduced proteolysis of C4b, similar to what observed for FH (see Figure 27).

As an additional mechanism of complement inhibition, C4BP can both impair formation of the CP/LP C3 convertase and accelerate dissociation of this complex into its component parts (see section 1.4.5). To evaluate the effect of PTX3 on these activities I performed a series of microtitre plate binding experiments, where AF conidia that had been pre-incubated with PTX3 were further incubated with a mixture of C4b, C2 and C4BP. In these experiments, C4b and C2 were used as components of the CP/LP C3 pro-convertase, in analogy with the experiments described above where C3b and FB were used as components of the AP C3 pro-convertase. Interestingly, C4b deposition onto AF conidia was inhibited rather than increased by PTX3 (Figure 29D). This is different to what observed when similar experiments were performed using C3b, FB and FH (see Figure 27B), and strongly suggests that the PTX3-dependent recruitment of C3 convertase components onto the AF fungal wall is limited to the AP, more specifically to C3b.

4.9 ROLE OF C3B AND FH IN THE PRO-PHAGOCYTIC ACTIVITY OF PTX3

Based on findings from microtitre plate binding and SEC experiments (see Figures 27 and 28, respectively) and the observation that PTX3 controls the balance between C3b and iC3b on AF conidia (i.e., by enhancing the binding of C3b and inhibiting that of iC3b, see Figure 23B and C), I hypothesized that C3b and FH could be sufficient to support the PTX3-dependent phagocytosis of AF conidia. Therefore, I evaluated the effect of PTX3 on AF phagocytosis by human PMNs replacing NHS, as a source of complement, with different combinations of C3b, FH and FI (where the latter was included to assess potential contributions by iC3b). Interestingly, PTX3 amplified conidia internalization and phagocytosis only when mixtures containing both C3b and FH were applied (Figure 30). The contribution of FI in this regard was dispensable, since, when supplemented with FI, mixtures of C3b and FH retained the PTX3 activity and this was lost when combinations of C3b and FI (i.e., without FH) were applied. Furthermore, C3b alone could not support the pro-phagocytic properties of PTX3, which strictly required FH. Taken together, these results indicate that PTX3 cooperates with FH to enhance opsonisation of AF by C3b and this facilitates recognition and phagocytosis of the C3b-opsonised conidia by PMNs.

To further investigate the contribution of C3b and FH to the pro-phagocytic activity of PTX3, I performed phagocytosis experiments *in vitro* using, as a source of complement and PMNs, the whole blood of mice that had been genetically depleted of selected complement components (i.e, C3 and FB) and, as a control, PTX3. In these experiments, FITC-labeled conidia were either incubated with PTX3 only or sequentially incubated with PTX3 and a mixture of C3b and FH, prior to addition of murine whole blood. PP was assessed by flow cytometry as the percentage of PMNs (Ly6G⁺CD11b⁺CD45⁺ cells) that had phagocytosed AF conidia (FITC⁺), based on an established protocol [2]. As shown in

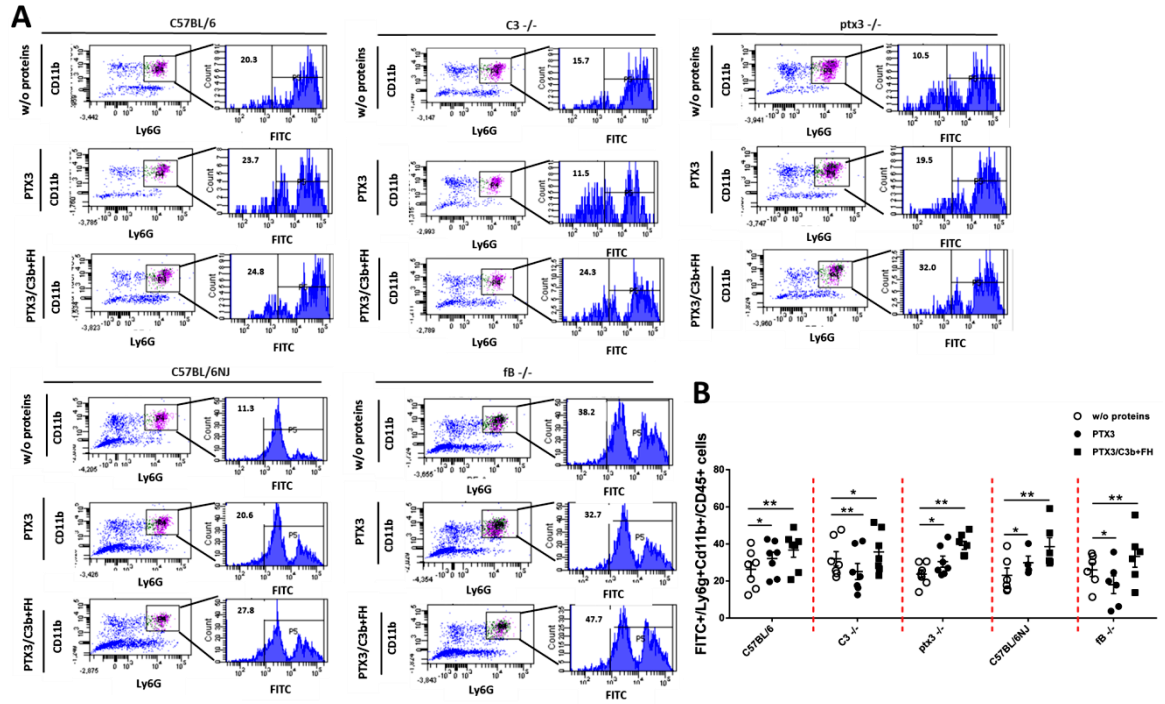


FIGURE 31. C3b and FH are necessary and sufficient for the PTX3-dependent opsono-phagocytosis of AF conidia by murine PMNs. FITC-labelled AF conidia were either incubated with PTX3 only (PTX3) or sequentially incubated with PTX3 and a mixture of C3b and FH (PTX3/C3b+FH), prior to addition of whole blood from *ptx3*^{-/-}, *C3*^{-/-} and *fB*^{-/-} deficient mice or their wild type littermates (C57BL/6 for *ptx3*^{-/-} and *C3*^{-/-}, and C57BL/6NJ for *fB*^{-/-} mice). PMNs were stained with fluorescently labeled α -CD45, α -Ly6G and α -CD11b antibodies, and percentage of FITC-positive PMNs (PP) was assessed by flow cytometry. 2 independent experiments were performed with a total number of 6 to 7 mice per experimental group. **A)** FACS gates and histograms from a representative experiment are shown, with numbers indicating the percentage of FITC-positive PMNs. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in panel **A**), and reported in the form of whisker (mean \pm SD) plots in **B**. Overlaid are markers representing the mean of duplicate PP observations from same animal in the absence of exogenous proteins (open circles), and presence of either PTX3 only (solid circles) or PTX3, C3b and FH (closed squares) (****P<0.0001, **P<0.01, *P<0.05; one-way ANOVA with Dunnett's multiple comparison test as post-hoc analysis).

Figure 31, in the blood of wild-type mice (C57BL/6 background for C3- and PTX3-deficient animals, and C57BL/6NJ background for FB-deficient animals), phagocytosis of AF conidia by PMNs significantly increased following pre-opsonisation with PTX3 (as compared to no added protein), and was further augmented when a mixture of C3b and FH was added. The pro-phagocytic effect of PTX3 was lost both in the C3- and FB-deficient blood, which lacks C3b due to the absence of the AP C3 convertase. However, the PTX3 activity was rescued when AF conidia were incubated with exogenous C3b and FH prior to infection. These findings are consistent with the previous observation that C3b and FH are sufficient to support the pro-phagocytic properties of PTX3 (Figure 30). Furthermore these properties are likely mediated by common mechanisms in human and murine PMNs (see Figures 30 and 31). Consistent with previous reports [2], exogenous human PTX3 potentiated phagocytosis of AF by PMNs in murine PTX3-deficient blood, and this effect was increased by mixtures of C3b and FH. It is worth noting here that the murine and human PTX3 proteins share 82% identical and 92% conserved amino acids, and both have been used in several murine models of disease with identical functional effects [346]. Also, the murine and human FH proteins share 63% sequence identity (up to 79% in the individual CCP domains), which suggests that they have similar structure/function relationships within the complement system [347]. To further strengthen this point, the regulatory regions (including the C3b binding sites) of the murine FH have been mapped in the same CCPs as in the human paralog, namely CCPs 1–5 and CCPs 18–20 [348]. Furthermore, human FH is a relatively abundant plasma protein with an average concentration of 233–320 $\mu\text{g/mL}$ (1.5–2.1 μM). Data on murine FH plasma levels are less clear, however it is believed that these are similar to those of the human protein [349,350].

4.10 ROLE OF COMPLEMENT RECEPTORS

The involvement of complement receptors in recognition and phagocytosis of complement-opsonised microbes and microbial moieties by phagocytic cells is well established [351]. Among those that interact with C3 fragments (other than the anaphylatoxin C3a) and are present on the plasma membrane of human PMNs, CR1, binds C3b with much higher affinity than iC3b, whereas the hetero-dimeric complement receptors 3 and 4 (CR3 and CR4, respectively) preferentially interact with iC3b [352-354]. CR3 (and to a less extent CR4) have been described to translocate from secretory vesicles and granules to the phagocytic cup, where they promote engulfment and internalization of iC3b-coated

microbes/particles [355]. However, the role of CR1 as a truly phagocytic receptor is still disputed, with a prevailing hypothesis that it acts as a molecular “arm” on the cell surface that catches C3b-coated particles and conveys them to CR3/CR4 for effective phagocytosis [352]. This view is consistent with the structure of this integral transmembrane protein whose extracellular domain comprises a long array of 30 or more CCPs. In addition, CR1 acts as a cofactor for the FI-mediated cleavage of C3b to iC3b and, subsequently, C3c and C3dg, where these fragments are recognized by CR3, CR4 and (mostly on B lymphocytes) CR2 [356]. However, a direct role for CR1 in phagocytosis has been proposed in human macrophages and PMNs [357,355]. Regardless of the way the various complement receptors take part in this process, a tight functional cooperation has been described in several reports with Fc receptors (FcRs), with FcγRII (CD32) and FcγRIII (CD16) playing a prominent role in PMNs [2,358]. It is conceivable that CRs and FcRs form dynamic multi-molecular assemblies that act as cell membrane foci both in recognition and uptake of microbes as well as transduction of microbial sensing into intracellular signaling [359,104]. Furthermore, previous reports indicate that both CR3 and FcRs are involved in the PTX3-dependent opsono-phagocytosis of AF by PMNs [2].

My findings (as described above) point to a unusual mechanism for the pro-phagocytic activity of PTX3, where this pentraxin does not affect complement activation on AF conidia (see Figure 18) but rather promotes C3b binding to the conidial surface (see Figures 18, 27 and 28), where this requires FH (see Figures 20, 27 and 28) and is sufficient to enhance phagocytosis of AF by human (Figure 30) and murine (Figure 31) PMNs. Given the critical role of C3b in these activities (but not iC3b, which is actually inhibited by PTX3; see Figure 18), I envisaged that CR1 could be important for the proposed mechanism and characterized the contribution of selected CRs and FcRs. With this aim in mind, phagocytosis assays were carried out in the presence of a blocking antibody to CR1 or purified human C3b. As shown in Figures 32 and 33, the PTX3-dependent enhancement of AF phagocytosis was abolished in these conditions. Interestingly, neither blocking antibodies to the iC3b receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) nor purified iC3b had any effect in these experiments, consistent with the observation that FI (that is required to generate iC3b) is dispensable for the PTX3 activity (see Figures 32 and 33). Furthermore, targeting of the FcγRs CD16 and CD32 with appropriate blocking antibodies abolished the pro-phagocytic properties of the long pentraxin, in line with the

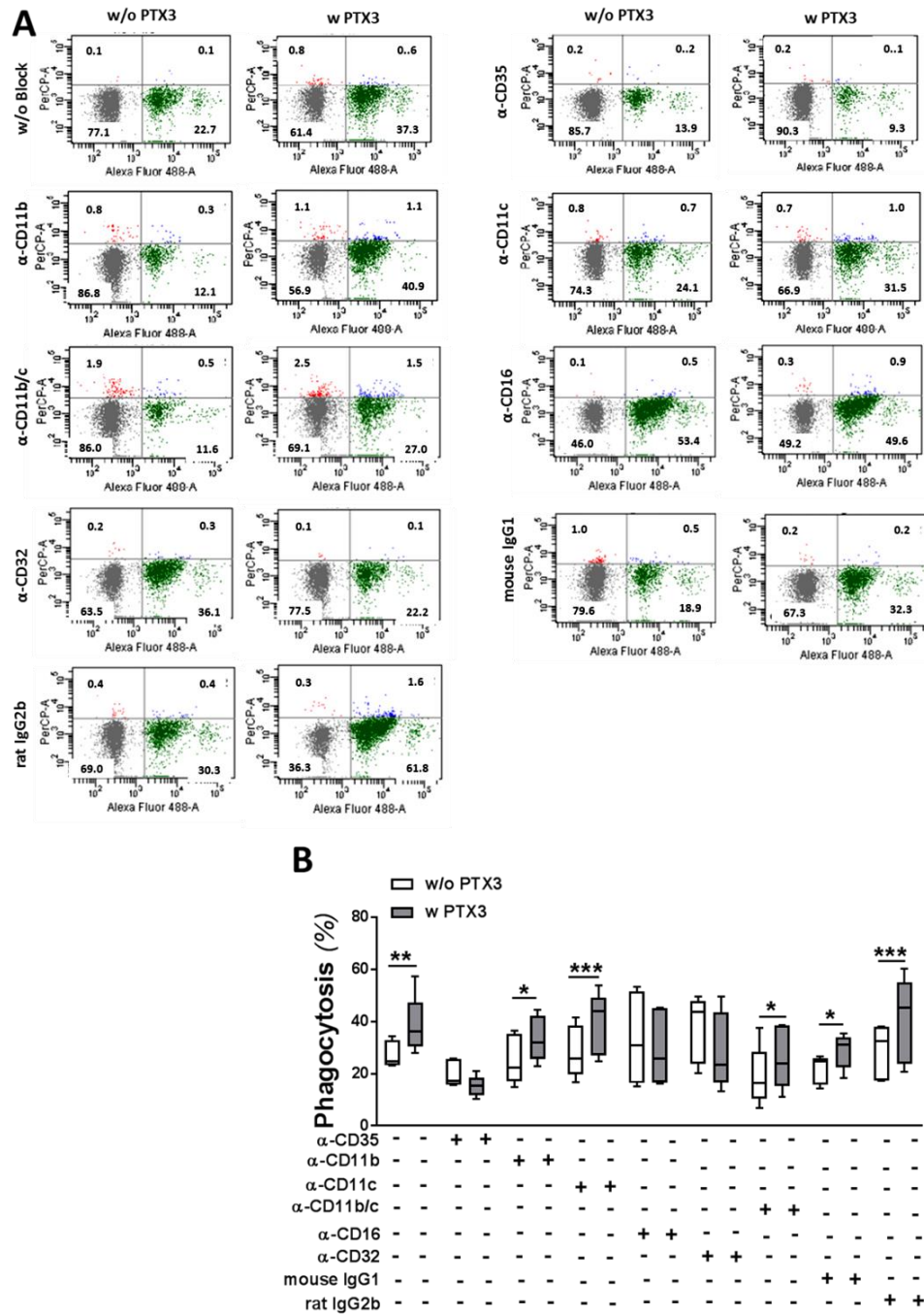


FIGURE 32. CR1 is required for the PTX3-mediated phagocytosis of AF conidia. Phagocytosis experiments were performed in the presence of 5% NHS and anti-CD35 (CR1), anti-CD11b (CR3), anti-CD11c (CR4), anti-CD16 (FcγRIII) or anti-CD32 (FcγRII) blocking antibodies (or control IgGs). **A)** FACS plots from a representative experiment (out of 5) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel A), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. 5 independent experiments performed in duplicate, each on a different donor (5 donors in total) (**P<0.001, *P<0.01, P<0.05, Student's paired t test).

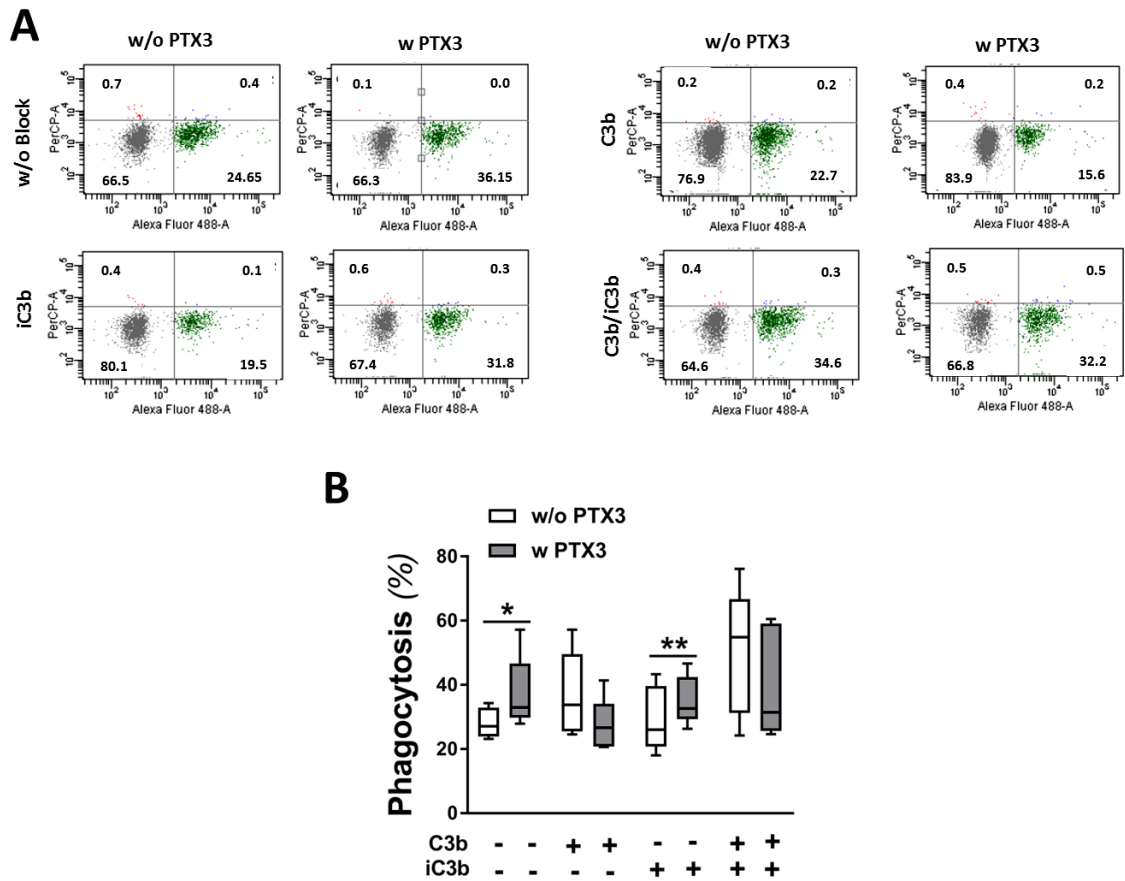


FIGURE 33. CR1 is required for the PTX3-mediated phagocytosis of AF conidia. Phagocytosis experiments were performed in the presence of C3b and iC3b (ligands of CR1 and CR3/4, respectively). **A)** FACS plots from a representative experiment (out of 4) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants in panel **A**), and reported in the form of box and whisker plots. White and grey boxes represent absence and presence of PTX3, respectively. 4 independent experiments performed in duplicate, each on a different donor (4 donors in total) (** $P < 0.01$, * $P < 0.05$, Student's paired t test).

evidence that Fc γ Rs are involved in the mobilization and activation of complement receptors (including CR1) in the phagocytic cup [104]. Isotype controls had no influence on PTX3 activity. These findings indicate that CR1, as major receptor of C3b, recognizes the PTX3-bound C3b on AF conidia and facilitates their uptake by PMNs, thus being essential for the pro-phagocytic properties of PTX3. Therefore, in the applied experimental conditions, CR1 acts as a truly phagocytic receptor and does so in a functional cooperation with Fc γ Rs, where it presumably participates in the intracellular signalling that mediates the ingestion of complement-opsonised particles [355].

4.11 STRUCTURE/FUNCTION OF PTX3 IN THE OPSONO-PHAGOCYTOSIS OF AF

A major aim of this study was to elucidate the structure/function relationships of the long pentraxin PTX3 in the opsono-phagocytosis of AF. In this regard, I described that the full-length PTX3 protein inhibits the binding of iC3b to AF conidia while promoting that of C3b (see Figure 18), and that C3b is sufficient (in conjunction with FH) to support the opsono-phagocytic activity of this long pentraxin (see Figures 30 and 31). Also, I presented data that clearly indicate that the N-terminal domain of PTX3 is the dominant AF binding region of this protein (see Figure 21 and Table 1). Furthermore, when applied as an isolated recombinant construct (N_PTX3), this domain recapitulated the inhibitory properties of the full-length protein towards FH with respect both to binding to and co-factor activity on AF conidia (see Figures 25 and 27). However, it failed to promote C3b deposition onto AF (see Figure 27B) and, more importantly, has been reported to lack pro-phagocytic activity towards AF [2]. I clarified this seemingly contradictory point by investigating the structure/function relationships of PTX3 in opsono-phagocytosis of AF conidia. To this end, I defined the structural units of the PTX3 protein that supports phagocytosis of AF as well as binding to and inhibition of the FH function on fungal conidia.

To this end, mutant constructs were used that have different quaternary structures in the context of both full length protein (PTX3-2S and PTX3-5S, which make tetramers and dimers, respectively, as compared to the octameric wild type PTX3 [298]) and its N-terminal domain (N_PTX3-3S, which forms dimers, as opposed to wild type N_PTX3 that assembles into tetramers [297]; see schematic drawings in Figure 34A). The interaction of these proteins with AF conidia was characterized using the QCM strategy described above (see Figure 22). In this regard, AF conidia were adsorbed onto cell compatible sensor chips and PTX3 proteins were injected at a range of concentrations (0.8 to 80 nM_{eq}). Global fitting of the recorded sensorgrams to a 1:1 Langmuir binding model allowed measurement of both the kinetic rate constants (k_{on} and k_{off}) and the equilibrium dissociation constant (K_D). As shown in Table 2, conidia were recognized by PTX3-2S, PTX3-5S and N_PTX3-3S, in addition to PTX3 and N_PTX3 with similar (high) affinities (K_D values of 1.85 ± 0.04 , 1.05 ± 2.13 and 2.86 ± 0.07 nM, respectively, as compared to 0.669 ± 0.001 and 3.72 ± 0.12 nM for PTX3 and N_PTX3, respectively). Furthermore, as was found for the wild type PTX3 protein (Table 2), the dissociation of these constructs from conidia was

remarkably slow (with k_{off} values of $1.66 \pm 0.34 \times 10^{-4} \text{ s}^{-1}$, for PTX3-5S, to $7.57 \pm 0.144 \times 10^{-4} \text{ s}^{-1}$, for PTX3-2S), indicating that the corresponding complexes with AF conidia were particularly stable under the applied experimental conditions. These findings corroborate what was described above in that stabilization of the PTX3/AF complex is mostly contributed by the N-terminal domain of the protein. However, consistent with the data shown in Figure 22, recognition of AF (i.e., complex formation/association) appears to be contributed by both domains, which might explain the minor though appreciable reduction in the measured k_{on} values (compared to that of PTX3, $1.08 \times 10^6 \pm 7.42 \times 10^{+2} \text{ M}^{-1} \text{ s}^{-1}$) when the C-terminal domain was deleted (i.e., in N_PT3 and N_PT3-3S, $1.33 \times 10^5 \pm 22.2$ and $2.20 \times 10^5 \pm 1.10 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$, respectively) or present in a reduced copy number (i.e., in PTX3-2S and PTX3-5S, $4.08 \times 10^5 \pm 24.7$ and $1.58 \times 10^5 \pm 33.1 \text{ M}^{-1} \text{ s}^{-1}$, respectively). Furthermore, dimers of the N-terminal domain (N_PT3-3S) retained full binding capacity, indicating that the AF-binding sites in this domain are either in individual protomer subunits or protomer pairs [297,321].

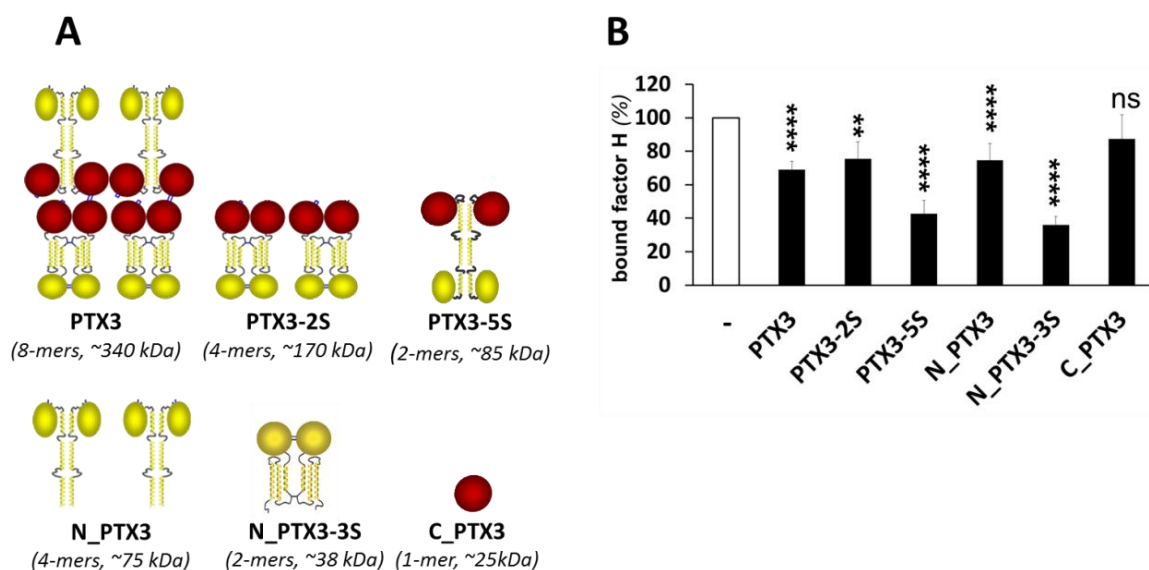


FIGURE 34. Dimers of the N-terminal domain of PTX3 (N_PT3-3S) inhibits the binding of FH to AF conidia. **A)** Schematic drawings of the PTX3, N_PT3 and C_PT3 proteins and their Cys/Ser substitution mutant constructs showing the corresponding oligomeric state and molecular weight. The N- and C-terminal domains are in yellow and red, respectively. **B)** In sequential incubation experiments in microtitre plates, AF conidia were pre-incubated individually with the constructs shown in A ($60 \text{ nM}_{\text{eq}}$), prior to addition of FH (133 nM). Results are expressed as percentage of FH binding in the absence of the PTX3 proteins (3 independent experiments with 10 replicates ($n=30$); mean \pm SEM; **** $P<0.0001$, ** $P<0.01$ compared to no added PTX3 proteins; Student's t test).

I then assessed the effect of each PTX3 protein on the binding of FH to AF conidia. To this end, I performed sequential incubation assays on microtitre filter plates, and found that PTX3-2S, PTX3-5S and N_PTX3-3S, in addition to PTX3 and N_PTX3, inhibited the binding of FH to conidia; no such activity was observed for C_PTX3, consistent with this domain being poorly involved in the interaction with AF, at least when assessed under equilibrium conditions (see Figure 34B). Furthermore, we observed that PTX3-5S and N_PTX3-3S were stronger inhibitors of FH than both PTX3 and N_PTX3. This might be

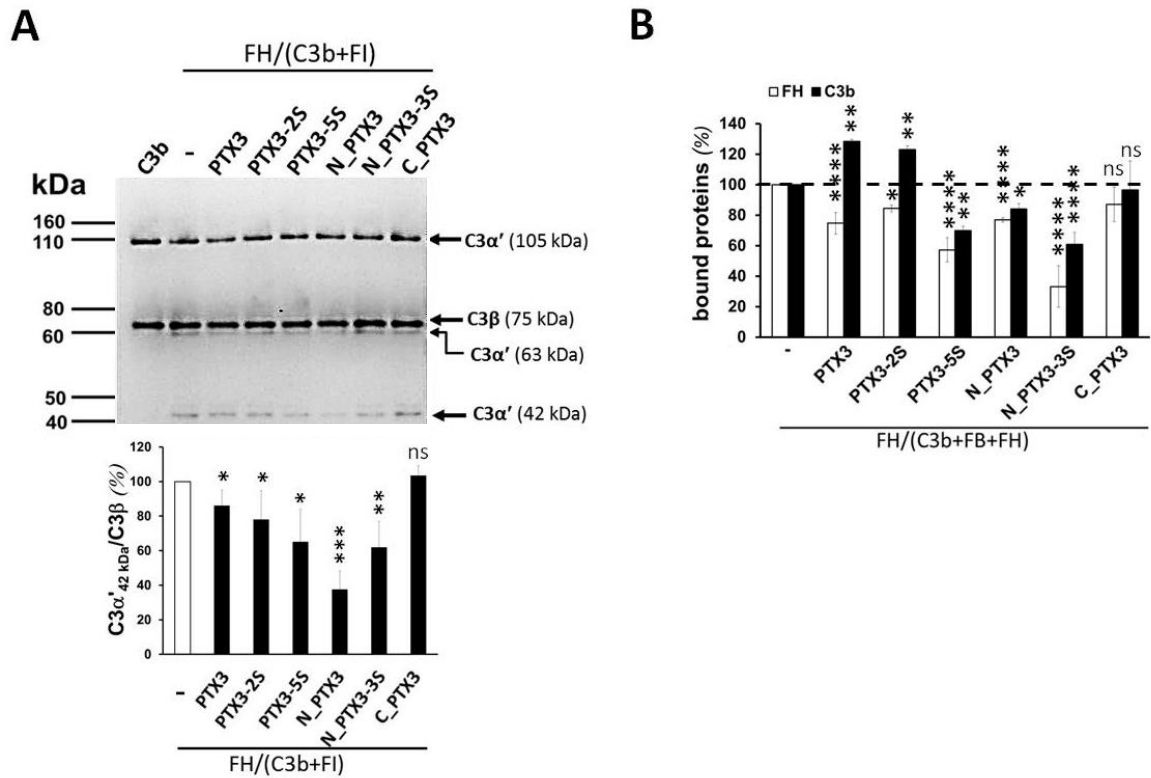


FIGURE 35. Structure/function of PTX3 in the regulation of the cofactor and AP C3 convertase decay-accelerating activity of FH on AF conidia. **A)** AF conidia were pre-incubated with either PTX3, PTX3-2S, PTX3-5S, N_PTX3, N_PTX3-3S, or C_PTX3, prior to addition of FH. Unbound proteins were removed, and a mixture of C3b and FI was applied. C3 fragments were revealed by western blotting using an α -C3 pAb as described in Figure 26A. Experiments performed in the absence of the PTX3 proteins (-) or FI (C3b) were taken as positive and negative controls, respectively. A representative gel is shown in the top panel. Blot images were analyzed by densitometry (lower panel), and results were expressed as percentage of the C3α' (42 kDa)/C3β intensity ratio in the absence of the PTX3 proteins (4 independent experiments performed in single; n=4, mean \pm SEM; ***P<0.001, **P<0.01, *P<0.05 compared to no added PTX3 proteins; Student's t test). **B)** Conidia were sequentially incubated with the PTX3 proteins and mixtures of C3b, FB and FH. Bound FH and C3b were revealed with the corresponding pAbs, and results are expressed as percentage of binding in the absence of the PTX3 proteins (3 experiments performed in triplicate; n=9, mean \pm SEM; ***P<0.0001, **P<0.01, *P<0.05; Student's t test).

due to these mutant constructs being smaller in size than the parent PTX3 protein (i.e., dimers vs octamers), which might allow a deeper penetration into the conidial wall (with increased occupancy of binding sites, as reported for N_PTX3 in Figure 22B). Also, despite of similar MW values (~85 vs ~75 kDa), PTX3-5S was more effective than N_PTX3 at inhibiting the binding of FH to AF conidia; probably, when the C-terminal domain is retained (i.e., in PTX3-5S as opposed to N_PTX3), this might stabilize the protein's interaction with fungal conidia (as suggested by our QCM data), and enhance competitive inhibition of FH. Taken together, these findings indicate that PTX3 mutant constructs containing the N-terminal domain recapitulate the binding and FH inhibiting activities of the full-length protein, and dimers of this domain (N_PTX3-3S) retain these functions.

Consistent with this, the mutants all inhibited the cofactor activity of FH on AF conidia (see gel image and the corresponding density plot in Figure 35A). However, when applied to an assay for the C3 convertase decay-accelerating activity of FH, only tetramers of the full length protein (PTX3-2S, that contains both N- and C-terminal domains; see Figure 34A), in addition to the octameric wild type PTX3, enhanced C3b binding to conidia, whereas the other constructs could not support this function, and rather caused inhibition of C3b (as well as FH) deposition on AF (Figure 35B). Pre-incubation of conidia with C_PTX3 had no effect on C3b and FH in this assay.

Prompted by the observation that tetramers of PTX3 (i.e., PTX3-2S) supported recruitment of C3b onto AF conidia (see Figure 35B), I assessed the phagocytic activity of this mutant and the other constructs in phagocytosis experiments *in vitro* and *in vivo*. As shown in Figures 36A and B, only PTX3-2S enhanced recognition and engulfment of FITC-labelled conidia by human PMNs *in vitro*, proving even more potent than the wild type protein (PP values of $58.2 \pm 12.9\%$ for PTX3-2S vs $32.7 \pm 8.5\%$ for PTX3). The dimeric PTX3-5S mutant and the N-terminal constructs N_PTX3 and N_PTX3-3S did not affect phagocytosis, despite of their ability to bind AF conidia and inhibit the co-factor activity of FH on their surface; the lack of pro-phagocytic activity of N_PTX3 is consistent with previous reports [2]. I then assessed the opsono-phagocytic properties of the PTX3-2S mutant *in vivo*, by adaptation of an animal model of AF phagocytosis previously developed in our laboratory [2]. C57BL/6 mice were intra-nasally challenged with FITC-labelled AF conidia that had been pre-opsonised with PTX3-2S (or PTX3, as a control), and sacrificed 4 hours post infection. AF phagocytosis by lung-recruited PMNs was assessed in the bronchoalveolar lavage (BAL) by flow cytometry using antibodies that recognize CD45

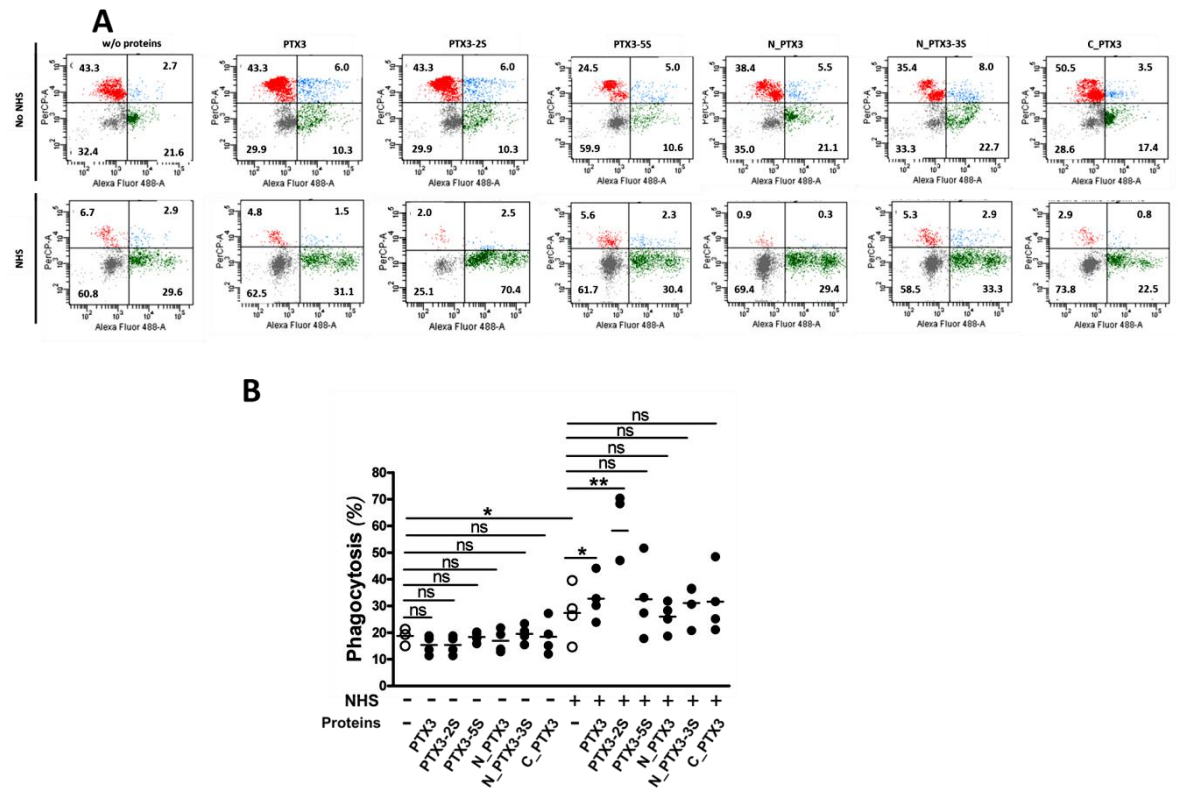


FIGURE 36. Tetrameric mutants of PTX3 (PTX3-2S) recapitulate the pro-phagocytic activity of the full length protein *in vitro*. **A)** Purified human PMNs were challenged with FITC-labelled AF conidia that had been pre-incubated with 3 nM_{eq} of either PTX3, PTX3-2S, PTX3-5S, N_PTXX, N_PTXX-3S, or C_PTXX at a [conidia]:[PMNs] ratio of 8:1, in the presence or absence of 5% NHS. Phagocytosis was assessed by flow cytometry as described in Figures 11-14, 31 and 32, (see section 3.12). **A)** FACS plots from a representative experiment (out of 4) are shown, with numbers indicating the percentage of PMNs in each quadrant. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in the lower right quadrants of panel A). Markers represent the mean of duplicate PP observations from same donor in the absence (open circles) and presence (closed circles) of the indicated PTX3 proteins. The overlaid horizontal bar represent the mean. 4 independent experiments performed in duplicate, each on a different donor (4 donors in total) (**P<0.01, *P<0.05, Student's paired t test).

(also known as leukocyte common antigen, LCA) and Ly6G (lymphocyte antigen 6 complex locus G6D). As shown in Figures 37 A and B, the percentage of FITC, CD45 and Ly6G triple positive cells (PMNs that had phagocytosed AF conidia) increased when conidia were pre-opsonised with PTX3-2S (and PTX3, as a control). This is consistent with my *in vitro* evidence (see Figures 36A and B) and indicates that tetramers of PTX3 (i.e., PTX3-2S) recapitulates the opsono-phagocytic activity of the octameric wild type PTX3.

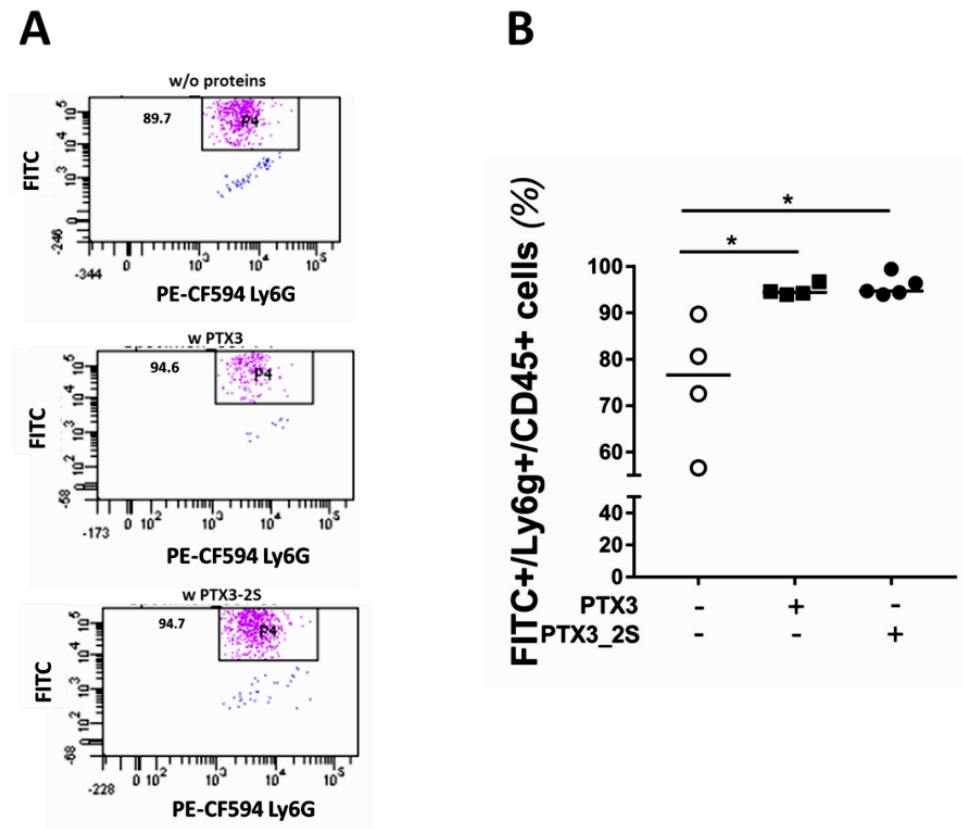


FIGURE 37. Tetrameric mutants of PTX3 (PTX3-2S) recapitulate the pro-phagocytic activity of the full length protein *in vivo*. 5×10^7 FITC-labelled AF conidia were pre-incubated with either PTX3-2S, PTX3 (both at 60 nM) or buffer, and intra-nasally injected into C57BL/6 mice. Animals were sacrificed 4 hours post infection, and PMNs from BALs were collected and stained with α -CD45 and α -Ly6G fluorescently labelled antibodies. Percentage of FITC positive PMNs was assessed by flow cytometry. **A)** FACS plots from a representative experiment (out of 2) are shown, with numbers indicating the percentage of FITC-positive PMNs. **B)** Results from all experiments are expressed as PP values (corresponding to the percentage values in panel A). Markers represent the mean of duplicate PP observations from same donor in the absence (open circles) and presence (closed circles) of the indicated PTX3 proteins. The overlaid horizontal bar represent the mean. (2 independent experiments were performed with a total number of 4-5 mice per experimental group, * $P < 0.05$; one-way ANOVA with Dunnett's multiple comparison test as post-hoc analysis).

These findings further support the hypothesis that the opsono-phagocytic properties of PTX3 have their basis in the selective recruitment of C3b (a major complement opsonin) rather than inhibition of FH co-factor activity (with concomitant reduction of iC3b).

5. DISCUSSION

In this study I provided a comprehensive characterization of the molecular mechanisms underlying the crosstalk between two major classes of soluble PRMs, namely the complement system and pentraxins, with specific regard to the AP and the long pentraxin PTX3, in the immune response to *A. fumigatus*. I found that PTX3 promotes the selective recruitment of C3b onto the conidial wall, by exclusively targeting the AP. FH is required for such process, thus pointing to a novel function (activating rather inhibitory) of this complement regulator when combined with PTX3. Also, CR1 recognizes the PTX3-bound C3b on AF conidia and facilitates their uptake and killing by PMNs. Therefore, I identified a novel functional axis involving factor H, C3b and CR1 that supports the pro-phagocytic and pro-killing activities of PTX3 in antifungal immunity.

Current literature indicates that PTX3 exerts pro-opsonic and pro-phagocytic activities against *A. fumigatus*, in that it recognizes this pathogen, and promotes phagocytosis and killing of fungal conidia by PMNs in a tight functional cooperation with the complement system [2]. In this regard, complement is long known to be essential for recognition and disposal of *A. fumigatus* [238,360], and the three pathways of complement activation, although to different extents, are all involved in the early immediate response to this fungal pathogen [334,181]. Previous reports from my own laboratory indicate that the AP is necessary to support the opsono-phagocytic activity of PTX3 [2]. Here, based on original experimental approaches (i.e., trypan exclusion flow cytometry-based phagocytosis assays and quantitative analyses of complement activation/deposition by western blotting) I provided further evidence to support this view. Of note, PTX3 has been shown to cooperate with MBL (i.e., major recognition component of the LP) in the recruitment of C1q (i.e., initiator of the CP) onto *A. fumigatus*, thus triggering cross-activation of the complement system on fungal conidia [191]. However, C1q has been reported to be dispensable for the pro-phagocytic activity of PTX3 both *in vitro* [2] and *in vivo* [141], and ablation of C4 (that is required for formation of the CP/LP C3 convertase) does not affect the PTX3-dependent phagocytosis of *A. fumigatus* by human PMNs [2]. Furthermore, although not required for PTX3 activity, in my hands both CP and LP prevailed over AP in terms of relative contribution to phagocytosis of *A. fumigatus* conidia (see section 4.2), in agreement with recent observations [181]. Therefore, following on available evidence from literature and my own findings, I went on to investigate and characterize the molecular mechanism underlying the crosstalk between PTX3 and the complement system in the

opsono-phagocytosis and killing of *A. fumigatus*. In particular, I tackled the individual components of the AP, and found that FH is required for the pro-phagocytic and pro-killing activities of PTX3. This was somewhat unexpected, given the current opinion that recruitment of FH on fungal conidia is a complement evasion strategy of *A. fumigatus* [246]. Even more surprising, in this regard, was our observation that FH promoted rather than inhibited phagocytosis and killing of the mold, where both processes were indeed augmented by PTX3. Given that in the same experimental conditions FI too enhanced phagocytosis of *A. fumigatus*, this might be explained by the notion that both FH and FI are required for the generation of iC3b on the conidial surface, where iC3b is a major opsonin that is mostly recognized by CR3 and CR4 [202]. In spite of this, PTX3 retained its pro-phagocytic properties in the absence of FI, which prompted me to investigate the PTX3-FH-*A. fumigatus* interaction and its functional implications.

In ELISA and flow cytometry experiments I observed a dose-dependent and saturable binding to conidia of both PTX3 and FH, in agreement with previous reports [2,82]. Furthermore, I developed a new QCM strategy that allowed real-time and label-free analysis of the interaction of both proteins with intact *A. fumigatus* conidia. This provided a detailed picture of kinetics and affinity, and unambiguously defined the relative contribution of the N- and C-terminal domains of PTX3 to the protein's interaction with *A. fumigatus*. Moreover, both in ELISA and QCM settings I found that PTX3 (via its N-terminal region) inhibited the interaction of FH with fungal conidia, likely through competition for common binding sites on the conidial wall. As expected, this resulted in inhibition of the cofactor activity of FH (i.e., decreased amounts of conidia-bound iC3b). Also, I observed that pre-opsonisation of *A. fumigatus* conidia with PTX3 caused a seeming reduction in the AP C3 convertase inhibiting/decay-accelerating activities of FH (i.e., increased amounts of conidia-bound C3b). However, at a closer inspection, FB (a component of the AP C3 convertase) was found to be dispensable for the PTX3-dependent recruitment of C3b onto *A. fumigatus* conidia, indicating that this phenomenon could not be ascribed to enhanced stabilization of the AP C3 convertase (which would have been an obvious consequence of PTX3 being an inhibitor of the binding of FH to the fungal spores). Consistent with this, pre-incubation of *A. fumigatus* with N_PTX3 (that strongly inhibited both conidia binding and cofactor activity of FH) failed to promote the recruitment of C3b. Furthermore, the PTX3-dependent recruitment onto *A. fumigatus* conidia of C3 fragments was restricted to C3b, with no involvement of intact C3 and iC3b. Importantly, this could not be ascribed to formation on the conidial surface of a

PTX3/FH/C3b ternary complex [315], since PTX3 concomitantly inhibited FH binding to AF conidia. I further addressed this point in both solid and fluid phase binding experiments and found that a pre-encounter of FH and C3b was required in order for C3b to interact with PTX3 both in solution and on the conidial surface. Therefore, I speculate that when in a complex with FH, C3b exposes/gains binding sites for PTX3, which mediate its recruitment (via binding to PTX3) to *A. fumigatus* conidia. This would imply a conformational change in the C3b protein that is induced by the interaction with FH, an occurrence, which has been documented in previous structural investigations [1]. Also, I propose that the tripartite PTX3-FH-C3b interaction evolves to a stable binary PTX3/C3b complex (that is able to bind AF conidia) via a transient PTX3/C3b/FH ternary complex, which loses FH. Formation of such a ternary complex has been described in a previous study from my laboratory [315], however its transition to a stable binary PTX3/C3b assembly has never been documented, and represents an novel and original hypothesis stemming out of my study. We extended my investigations to C4BP, the major soluble inhibitor of both CP and LP, and found that PTX3 (again, via its N-terminal domain) inhibited the binding to *A. fumigatus* of this complement regulator as well as its cofactor activity on fungal conidia. However, the long pentraxin could not promote the recruitment of C4b, as opposed to what we observed with FH and C3b. This further supports the view that is indeed the AP of complement that is primarily targeted by PTX3.

Quantitative western blotting analysis of proteolysis and deposition of C3 on *A. fumigatus* conidia provided additional evidence that PTX3 tips the balance between C3b and iC3b in favour of the former, especially at early times in the course of complement activation. No effect was observed, instead, on C4 and its fragments, consistent with CP and LP being unnecessary for the pro-phagocytic activity of PTX3. Interestingly, the long pentraxin did not enhance the rate of cleavage of C3 (and C4), neither did it increase binding to conidia of the entire population of C3 (and C4) fragments. Given that PTX3 inhibits the interaction of *A. fumigatus* with iC3b (that cannot form the AP C3 convertase) and promotes that of C3b (that acts as scaffold for formation of the AP C3 convertase), this is counterintuitive, and raises the question as to whether PTX3-bound C3b is still accessible to FB for formation of the AP C3 convertase. This is an important point that might have general implications beyond anti-fungal immunity, in situations where the PTX3-FH-C3b axis is likely to play a biological role, for example atherosclerosis [361] and age-related macular degeneration [285].

Prompted by the observation that PTX3 shapes the balance between C3b and iC3b on *A. fumigatus*, I addressed the role of the PTX3-FH-C3b axis in the opsono-phagocytosis of fungal conidia, and provided evidence that C3b and FH are sufficient to support the pro-phagocytic activity of PTX3 both in human and murine PMNs *in vitro*. In this regard it is worth noting that, in an attempt to define the structural determinants of this protein's function, I found that only PTX3-2S (a PTX3 mutant with tetrameric quaternary structure) could enhance both C3b binding to conidia (in a FH-dependent manner, like the wild type protein) and phagocytosis of the fungal spores by human PMNs *in vitro* or murine PMNs *in vivo*. This observation further supports the view that it is the recruitment of C3b on *A. fumigatus* that mediates the pro-phagocytic role of PTX3, and this occurs only when FH is present.

Complement receptors are long known to be involved in recognition and phagocytosis of complement-opsonised microbes and microbial moieties [202]. C3b and iC3b are both endowed with opsonic activities, and are preferentially recognized by CR1 and CR3/CR4, respectively. These receptors are present on both human and murine PMNs [362], and have specificities for different sites on the C3 molecule [202]. I targeted selected complement receptors using blocking antibodies and purified C3b or iC3b (as competitors), and described that the PTX3-dependent enhancement of *A. fumigatus* phagocytosis by human PMNs was abolished *in vitro* when CR1 (but not CR3 or CR4) was blocked. This is in line with the hypothesis that PTX3 acts through promotion of C3b (but not iC3b) deposition on *A. fumigatus* conidia, and the notion that CR1 is a major cellular receptor for this complement opsonin [202].

Surprisingly, in my hands functional depletion of CR3 (and CR4) with a blocking antibody or iC3b (cognate ligand of CR3/CR4) had no effect on the pro-phagocytic activity of PTX3. In this regard, it is known that CR3 can directly recognize components of the microbial wall, and that phagocytosis via this receptor is most efficient when pathogens are coated with the complement fragment iC3b [363]. Probably, inhibition of iC3b (in conjunction with enhancement of C3b), as mediated by PTX3 on the conidial wall, biases the relative contribution to phagocytosis of *A. fumigatus* by complement receptors towards CR1. Furthermore, targeting of the Fc γ Rs CD16 and CD32 abolished the pro-phagocytic properties of the long pentraxin, in line with the evidence that Fc γ Rs are involved in the mobilization and activation of complement receptors (including CR1) in the phagocytic cup [104]. The manner whereby Fc γ Rs and complement receptors interact remains to be

elucidated. It has been suggested that cross-linking of Fc γ R leads to activation of the Src family kinases and stimulation of phospholipase C- γ (PLC γ) [364]. The release of diacylglycerol from phosphoinositides would then activate protein kinase C (PKC), which would in turn release selected integrins (including CR3 and CR4) from their cytoskeletal anchorage and prime them for activation. Novel isoforms of PKC, PKCs δ and/or ϵ , are likely to be prominent in this event, inasmuch as diacylglycerol, but not calcium elevation, is required for CR3 activation. Indeed, these PKC isoforms have been attributed a prominent role in phagocytosis [365]. PKC may alter cytoskeletal structure via phosphorylation of F-actin-bundling substrates, such as myristoylated alanine-rich C kinase substrate (MacMARCKs) that are enriched on macrophages, which is involved in enhancing the mobility of β 2 integrins [366]. By acquiring lateral mobility as a result of cytoskeletal detachment, integrins could diffuse to sites of ligand accumulation, forming high avidity complexes. This mechanism might apply to the membrane receptor CR1 (that is not an integrin, as opposed to CR3 and CR4), thus explaining why in my hands it acted as a phagocytic receptor, in cooperation with the Fc γ Rs CD16 and CD32.

Given that my findings indicate that the pro-phagocytic properties of PTX3 are likely mediated by common mechanisms in human and murine PMNs (see Figures 30 and 31), it is important to highlight here difference and similarities between human and murine CR1 with regard to gene/protein structure, function and expression. The extracellular domain of human CR1 consists of an array of 30 or more homologous units (short consensus repeats, SCRs, or complement control proteins, CCPs) comprising of 60-65 amino acids. Distinctive for CR1 is the arrangement of the CCPs in three to six long homologous repeats (LHR) made up of seven CCP units and displaying 70-95% identity between each LHR. The transmembrane region of CR1 consists of 25 hydrophobic amino acids followed by four positively charged residues and the C-terminal cytoplasmic domain consists of 39 amino acids [367,368]. These CCPs have C3b and C4b binding activity, and the CR1 protein acts as a co-factor in the FI mediated-cleavage of these substrates [369]. Furthermore, the human gene is expressed on a number of cell types, including monocytes/macrophages, neutrophils, eosinophils, basophils, natural killer (NK) cells, B cells and a subset of T cells. Mice have the CD21/Cr2 gene that codes for two receptor proteins (i.e., CR1 and CR2), whose synthesis is under control of alternative splicing mechanisms (CR1 contains 6 additional CCP domains at its N-terminus compared to CR2) [370,371]. The mouse CR1 protein recognizes C4b and C3b, and possesses co-factor activity in the FI mediated cleavage of both fragments, similar to the human counterpart

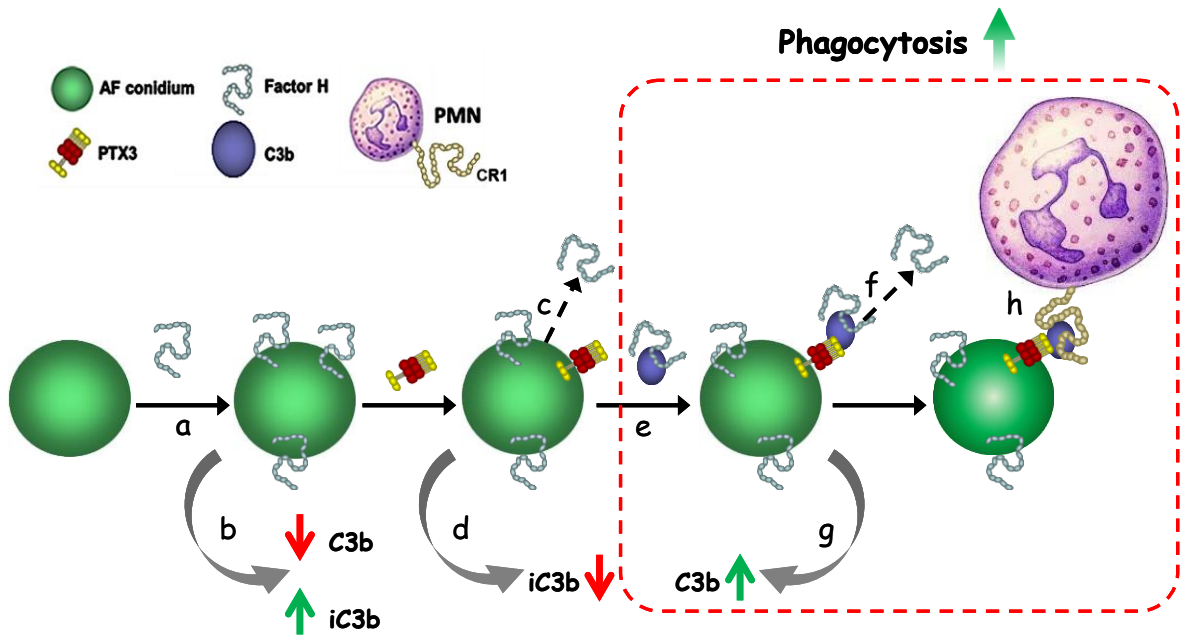


FIGURE 38. Proposed mechanism for the opsono-phagocytic activity of PTX3. *A. fumigatus* conidia recruit FH onto their surface (a), thus leading to reduced C3b and enhanced iC3b formation on the conidial wall (b). Whether this is truly a complement escape strategy is, however, questioned by our observation that FH promoted rather inhibited phagocytosis of *A. fumigatus* conidia. PTX3, via its N-terminal domain, shares with FH common binding sites on *A. fumigatus*, therefore acting as a competitive inhibitor of FH (c) and its cofactor activity (d). However, these inhibitory properties *per se* cannot explain the opsono-phagocytic activity of PTX3, since iC3b, its receptors CR3 and CR4, and FI (that, with FH as a cofactor, catalyzes the proteolysis of C3b to iC3b) were all dispensable in our *in vitro* phagocytosis experiments. In the course of AP activation/amplification FH recognizes C3b, and induces structural modifications in the C3b protein [1]. These might expose neo-binding sites for PTX3, which mediate formation on the conidial surface of a transient PTX3/C3b/FH ternary complex (e) that evolves into a more stable binary PTX3/C3b complex following dissociation of FH (f). In this way, more C3b is deposited onto *A. fumigatus* conidia (g) and presented to its cognate receptor on PMNs (i.e., CR1), which promotes recognition and phagocytosis of *A. fumigatus* (h). Our evidence suggests that it is the enhanced deposition of C3b (not the inhibition of FH and, subsequently, iC3b) that is responsible for the opsono-phagocytic activity of PTX3.

species the two genes are localized on chromosome 1. In addition, of relevance to my study, the CD21/Cr2 gene is expressed on murine neutrophils [371], besides B cells and follicular dendritic cells, where it is traditionally described [373].

A major aim of this study was to elucidate the structure/function relationships of the long pentraxin PTX3 in the opsono-phagocytosis of AF. In this regard, I demonstrated that PTX3 mutant constructs containing the N-terminal domain recapitulated the binding and

FH inhibiting activities of the full length protein, and dimers of this domain (N_PTX3-3S) retained these functions. I could not study isolated protomers of the N-terminal domain of PTX3, because this region of the protein only folds into dimers (when the Cys residues that form inter-chain disulfide bonds are replaced with Ser residues), which are stabilized by coil-coil non-covalent interactions [297]. Given that dimers may be the simplest organisation unit of PTX3, I could speculate that the minimal structural determinants of the inhibitory function of PTX3 towards FH on AF conidia are indeed dimers of its N-terminal domain. Furthermore, only tetramers of PTX3 (i.e., PTX3-2S) showed pro-phagocytic activity *in vitro* and *in vivo*. Therefore, I speculate that tetramers of the C-terminal domain (i.e., in the context of the PTX3-2S tetrameric mutant) are the minimal structural unit of PTX3 that retains C3b binding to and phagocytosis of *A. fumigatus* conidia. The N-terminal region (either in its tetrameric or dimeric forms) can therefore be regarded as a molecular “linker” in the interaction between conidia and phagocytosis-active tetramers of the C-terminal domain. Further investigations on the interaction of C3b with the two domains of PTX3 are, however, needed to support this hypothesis.

Based on our findings, I propose a mechanism for the opsono-phagocytic activity of PTX3 that is described in Figure 38. Given the prominent roles in the proposed mechanism for FH, C3 (in the form of C3b) and CR1, it would be interesting to assess if genetic variation in these complement components has an impact on the risk of IA, e.g. in immunocompromised individuals. This would be of great clinical relevance, with major regard to the management of bone marrow and solid organ transplantation, in that it would provide a broader genetic framework for selection of suitable donors.

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